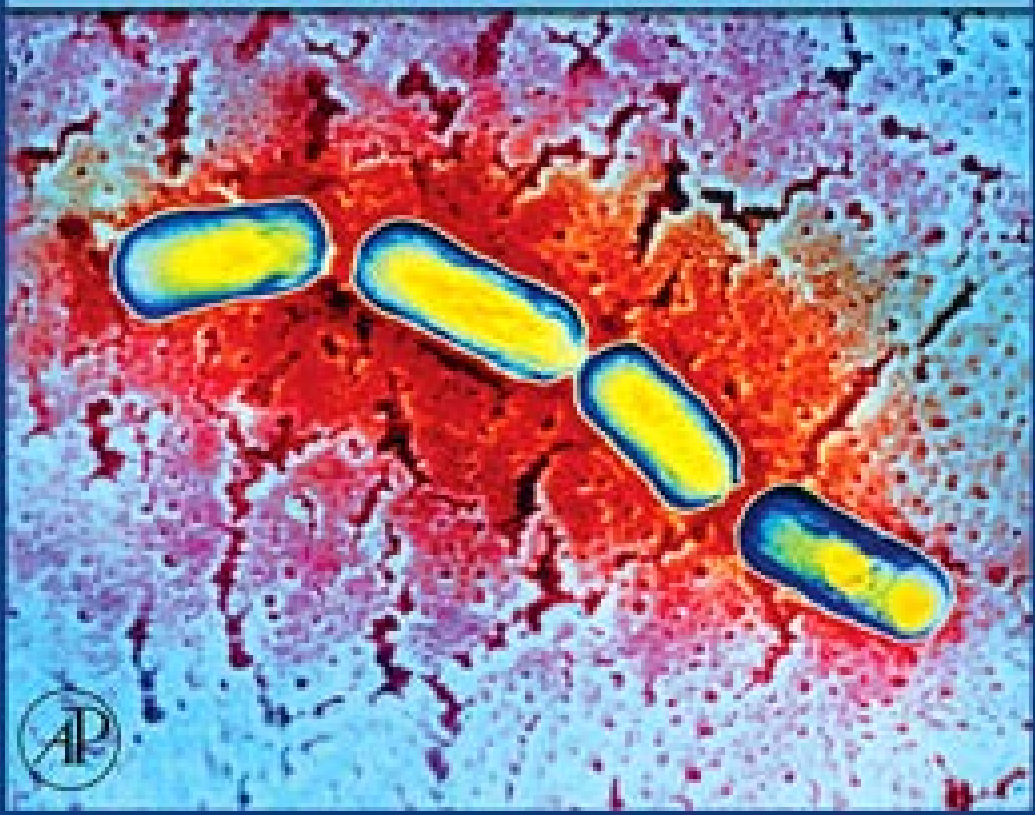
# The Comprehensive Sourcebook of BACTERIAL PROTEIN TOXINS

Third Edition

Edited by JOSEPH E. ALOUF and MICHEL R. POPOFF



# The Comprehensive Sourcebook of Bacterial Protein Toxins

# The Comprehensive Sourcebook of Bacterial Protein Toxins

THIRD EDITION

*Editors*

Joseph E. Alouf

*and*

Michel R. Popoff

*Institut Pasteur, Paris, France*

AMSTERDAM • BOSTON • HEIDELBERG • LONDON
NEW YORK • OXFORD • PARIS • SAN DIEGO
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO
Academic Press is an imprint of Elsevier

Academic Press is an imprint of Elsevier
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

This book is printed on acid-free paper. ♾

Front cover image: *Clostridium Bacteria, BSIP*
provided by Science Photo Library.



**Library of Congress Cataloging-in-Publication Data**
Application submitted

**British Library Cataloguing in Publication Data**
A catalogue record for this book is available from the British Library

ISBN 13: 978-0-12-088445-2
ISBN 10: 0-12-088445-3

For information on all Academic Press publications visit our Web site at
www.books.elsevier.com

Printed in the United States of America
05 06 07 08 09 10 9 8 7 6 5 4 3 2 1

# In Memoriam

During the making of this third edition of *The Comprehensive Sourcebook of Bacterial Protein Toxins*, Dr. Gianfranco Menestrina (Trento, Italy) unfortunately perished in a motorcycle accident on July 8, 2004. He was 50 years old. This has caused a deep loss and sadness for his family, friends, collaborators, and many of those who had any scientific exchange with him. Gianfranco Menestrina was always interested in membrane biophysics, membrane compounds, and associated regulation of osmotic compartments. He graduated in Physics in 1978 at the University of Trento, and had built his career (more than 95 published articles, many chapters of books, 30 graduated students, and 20 post-doctoral fellows) at the interface of Physics and Biology. In 2002, Gianfranco became the Director of the Section of Biophysics in Trento for the CNR Institute. He was a very active member of different Societies of Biophysics and Toxinology. He was awarded several times and received post-doctoral or visiting Professor Fellows at Universities of Bochum (G), London (UK), Wisconsin (USA), and La Habana (Cuba). His scientific interests swept across membrane-inserted polymers or peptides, with continual interest in pore-forming toxins. He was frequently chosen as a chairman in meetings and organized several meetings with recognized success.

Kind attention to anybody and caution in the language used were Gianfranco's marks. He provided a unanimous impression of an authentic man, of a true, honest, and rigorous scientist. Scientific collaboration with him and his lab was deeply fair, concerted, always sharing the goal of the best science of the moment with the environment provided. Presentation of a scientific problem or argument, an open mind, and explanations of experiments by him were always enriching, satisfying, or favoring scientific exchanges. Reliability, loyalty, friendship, fidelity, distinction, and a gentleman are words kept to the memory of Gianfranco.

Goodbye, Gianfranco; sympathies go to your family, and see you in a better world.

*Gilles Prévost and Collaborators*
*Joseph E. Alouf*
*Michel R. Popoff*

# Contents

## SECTION II: BACTERIAL PROTEIN TOXINS ACTING IN THE INTRACELLULAR COMPARTMENT OF EUKARYOTIC CELLS

## SECTION III: TOXINS ACTING ON THE SURFACE OF TARGET CELLS (EXCEPT SUPERANTIGENS)

# Contributors

**Acharya, K. Ravi**
Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

**Acquadro, Martin A.**
Department of Anaesthesia, Massachusetts General Hospital, Boston, MA 02114, USA

**Aktories, Klaus**
Institute of Pharmacology and Toxicology, Freiburg University, D-79104 Freiburg, Germany

**Alouf, Joseph E.**
Emeritus Professor, Pasteur Institute, Paris, France

**Arnold, Ralf**
Dept. of Medical Microbiology, Otto von Guericke University of Magdeburg, 29120 Magdeburg, Leipziger Str. 44, Germany

**Babon, Aurélie**
Département d'Ingénierie et d'Etudes des Protéines (DIEP) bat 152, CEA-Saclay, 91191 Gif sur Yvette Cedex, France

**Backert, Steffen**
Universität Klinikum, Institut für Medizinische Mikrobiologie, Otto-von-Guericke-Universität, D-39120 Magdeburg, Germany

**Baker, Matthew D.**
Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

**Barbieri, Joseph T.**
Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226, USA

**Basak, Ajit K.**
Department of Crystallography, Birbeck College, Malet Street, London WC1E 7HX, UK

**Bast, Darrin J.**
Department of Microbiology, Mount Sinai Hospital and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

**Billington, Stephen J.**
Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721, USA

**Bohnert, Stephanie,**
Molecular Neuropathobiology Laboratory, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

**Boquet, Patrice**
INSERM U627, Toxines Bactériennes dans la relation hôte-pathogènes, Faculté de Médecine, 28 Avenue de Valombrose, 06107, Nice Cedex 2, France

**Borodic, Gary E.**
Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Boston, MA 02114, USA

**Braun, Volkmar**
Microbiology, University of Tübingen, D-72076 Tübingen, Germany

**Bryant, Amy E.**
Infectious Diseases Section, Veterans Affairs Medical Center, Boise, ID 83702-4598, USA

**Carniol, Karen**
Department of Ophthalmology, Harvard Medical School and The Schepens Eye Research Institute, Boston, MA 02114, USA

**Carnoy, Christophe**
Université de Lille II, Institut de Biologie de Lille, 1 rue du Professeur Calmette, Lille Cedex, B.P. 245 -59021, France

**Chenal, Alexandre**
Département d'Ingénierie et d'Etudes des Protéines (DIEP) bat 152, CEA-Saclay, 91191 Gif sur Yvette Cedex, France

**Cole, Ambrose**
Defense Science and Technology Laboratory, Porton Down, Salisbury, Wilts SP4 0JQ, UK

**Colin, Didier A.**
Institut de Bactériologie de la Faculté de Médecine, 3, rue Koeberlé, F-67000 Strasbourg, France

**Collier, John**
Dept. of Microbiology and Immunology, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115, USA

**Cover, Timothy L.**
Departments of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37212, USA

**Dalla Serra, Mauro**
CNR-ITC, Istituto Di Biofisica Sezione di Trento, Via Sommarive, 18, I-38050 Povo (Trento) Italy

**de Azavedo, Joyce C. S.**
Department of Microbiology, Mount Sinai Hospital and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

**Deinhardt, Kartrin**
Molecular Neuropathobiology Laboratory, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

**Dobrindt, Ulrich**
Institut für Molekulare Infectionsbiologie, Röntgenring 11, D-97070 Würzburg, Germany

**D'Souza, Jocelyne M.**
School of Molecular and Microbial Biosciences (G08), University of Sydney, Sydney, NSW 2006, Australia

**Dubreuil, Daniel**
Faculty of Veterinary Medicine, Dept. of Pathology & Microbiology, University of Montreal, 3200 Rue Sicotte, J2S 7C6 Saint-Hyacinthe, Quebec, Canada

**Feil, Susanne C.**
Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, 9 Princes St., Fitzroy, Victoria 3065, Australia

**Flateau, Gilles**
INSERM U627, Toxines Bactériennes dans la relation hôte-pathogènes, Faculté de Médecine, 28 Avenue de Valombrose, 06107, Nice Cedex 2, France

**Fournier, Bénédicte**
Laboratoire des Listeria, Institut Pasteur, 25 Rue du Dr. Roux, Paris F-75015, France

**Franco, Augusto A.**
Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

**Frank, Dara W.**
Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226, USA

**Fraser, John D.**
Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Rd., Grafton, Auckland, New Zealand

**Frey, Joachim**
Institute of Veterinary Bacteriology, University of Berne, Laenggassstrasse 122, Bern CH–3001, Switzerland

**Frisan, Teresa**
Microbiology and Tumor Biology Center, Karolinska Institute Box 280, Stockholm S-171 77, Sweden

**Galmiche, Antoine**
Institüt für Medizinische Strahlenkunde und Zellforschung, Versbacher Str. 5, 97078 Würzburg, Germany

**Gauthier, Michel**
INSERM U627, Toxines Bactériennes dans la relation hôte-pathogènes, Faculté de Médecine, 28 Avenue de Valombrose, 06107, Nice Cedex 2, France

**Giddings, Kara S.**
Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, WAB 347, Boston, MA 02115, USA

**Gillet, Daniel**
Département d'Ingénierie et d'Etudes des Protéines (DIEP) bat 152, CEA-Saclay, 91191, Gif sur Yvette Cedex, France

**Gilmore, Michael S.**
Department of Ophthalmology, Harvard Medical School and The Schepens Eye Research Institute, Boston, MA 02114, USA

**Goebel, Werner**
LS Mikrobiologie Am Hubland, Theodor-Boveri-Institut, D-97074 Würzburg, Germany

**Granum, Per Einar**
Norwegian School of Veterinary Science, P.O. Box 8146, N-0033 Oslo, Norway

**Gurcel, Laure**
Dept. Microbiology and Molecular Medecine, University of Geneva, Faculty of Medicine, 1 rue Michel Servet, 1211 Geneva 4, Switzerland

**Hacker, Jörg**
Institut für Molekulare Infectionsbiologie, Röntgenring 11, D-97070 Würzburg, Germany

**Hertle, Ralf**
Microbiology-Membranephysiology, University of Tübingen, D-72076 Tübingen, Germany

**Hirst, Timothy R.**
School of Molecular and Microbial Biosciences (G08), University of Sydney, Sydney, NSW 2006, Australia

**Ho, Mangfei**
Department of Microbiology, University of Illinois at Urbana-Champaign, 601 S. Goodwin Avenue, B103 CLSL, Urbana, IL 61801, USA

**Holmes, Randall K.**
Dept. of Microbiology, Campus Box B-175, Univ. of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA

**Holmgren, Jan**
Dept. Med. Microbiol. Immunol., Göteborg University, Box 435, Göteborg S-41346, Sweden

**Horiguchi, Yasuhiko**
Department of Bacteriology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka, 565-0871, Japan

**Iacovache, Ioan**
Dept. Microbiology and Molecular Medicine, Centre Médical Universitaire, University of Geneva, 1 rue Michel Servet, 1211 Geneva 4, Switzerland

**Imanishi, Ken-ichi**
Department of Microbiology and Immunology, School of Medicine, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 152-8666, Japan

**Johannes, Ludger**
Laboratoire Trafic et Signalisation, UMR 144, Institut Curie, Paris Cedex 05, Paris F-75248, France

**Johnson, Arthur E.**
Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, Rm. 116 Reynolds Medical Bld., College Station, TX 77843-1114, USA

**Johnson, Eric A.**
Department of Food Microbiology and Toxicology and Bacteriology, University of Wisconsin, Madison, WI 53706, USA

**Jost, B. Helen**
Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721, USA

**Just, Ingo**
Department of Pharmacology and Toxicology, Medical School of Hannover, Carl Neuberg Strasse 1, D-0625 Hannover, Germany

**Kato, Hidehito**
Department of Microbiology and Immunology, School of Medicine, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 152-8666, Japan

**König, Wolfgang**
Medizinische Mikrobiologie, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany

**König, Brigitte**
Medizinische Mikrobiologie, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, D-39120 Magdeburg, Germany

**Lacharme, Lizeth**
Bacterial Molecular Pathogenesis Group, Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, Langford BS40 5DU, UK

**Ladhani, Shamez N.**
Department of Pediatrics, Newham General Hospital, Glen Road, London E13 8RU, UK

**Lamaze, Christophe**
Trafic et Signalisation, Institut Curie, UMR144, Paris Cedex 05, Paris F-75248, France

**Lemaitre, Nadine**
Université de Lille II, Institut de Biologie de Lille, 1 rue du Professeur Calmette, Lille Cedex, B.P. 245 -59021, France

**Lemichez, Emmanuel**
INSERM U627, Toxines Bactériennes dans la relation hôte-pathogènes, Faculté de Médecine, 28 Avenue de Valombrose, 06107, Nice Cedex 2, France

**Leppla, Stephen**
Bacterial Toxins and Therapeutics Section, National Inst. of Allergy & Infectious Diseases, NIH, Bethesda, MD 20892-4349, USA

**Lereclus, Didier**
Unité de Génétique Microbienne et Environnement, INRA, La Minière, Guyancourt F-78285, France

**Liu, George**
Division of Pediatric Infectious Diseases, 9500 Gilman Drive, Mail Code 0687, UCSD School of Medicine, La Jolla, CA 92093-0687, USA

**Locht, Camille**
INSERM U629, Institut Pasteur de Lille, 1 rue du Prof. Calmette, F-59019, Lille Cedex, France

**Ludwig, Albrecht**
Institut für Medizinische Mikrobiologie, Klinikum der Johann Wolfgang Goethe-Universität, Paul-Ehrlich-Str. 40, D-60596 Frankfurt am Main, Germany

**Mahillon, Jacques**
Laboratory of Food & Environmental Microbiology, UCL, Croix du Sud, 2/12, Louvain-la-Neuve, Belgium

**Maresso, Anthony W.**
Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226, USA

**Masignani, Vega**
IRIS Research Center, Chiron Vaccines, Via Fiorentina, 1, 53100 Siena, Italy

**Masin, Jiri**
Cell and Molecular Microbiology, Videnska 1083, Institute of Microbiology, Czech Academy of Sciences, Prague 142 20, Czech Republic

**McClain, Mark**
Division of Infectious Diseases, A3310 Medical Center North, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

**McClane, Bruce**
Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, E1240 BST, Pittsburgh, PA 15261, USA

**Mekada, Eisuke**
Dept. of Cell Biology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan

**Melton, Jody**
Dept. of Microbiology, University of Oklahoma Health Science Center, 940 Stanton L. Young Boulevard, Oklahoma City, OK 73104, USA

**Menestrina, Gianfranco**†
CNR-ITC, Via Sommarive, 18, I-38050, Povo (Trento), Italy

**Ménez, André**
Département d'Ingénierie et d'Etudes des Protéines, (DIEP) bat 152, CEA-Saclay, 91191, Gif sur Yvette Cedex, France

**Michelet, Nathalie**
Laboratory of Food & Environmental Microbiology, UCL, Croix du Sud, 2/12, Louvain-la-Neuve, Belgium

**Mitchell, Tim J.**
Division of Infection and Immunity, University of Glasgow, Glasgow G12-8QQ, UK

**Miyoshi, Shin-ichi**
Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700-8530, Japan

**Miyoshi-Akiyama, Tohru**
Department of Infectious Diseases, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

**Molgo, Jordi**
Laboratoire de Neurobiologie Cellulaire et Moléculaire, UPR 9040, Institut Fédératif de Neurobiologie, CNRS 32-33, Gif sur Yvette F-91198, France

**Monteil, Henri**
Institut de Bactériologie de la Faculté de Médecine, 3 rue Koeberlé, F-67000 Strasbourg, France

**Mourey, Lionel**
Institut de Pharmacologie et de Biologie Structurale du CNRS, UMR 5089, Groupe de Biophysique Structurale, 205 route de Narbonne, F-31077, Toulouse Cedex, France

**Müller-Alouf, Heide**
Domaine de la Ronce, 7 Avenue des Cèdres, Ville d'Avray F-92410, France

**Nizet, Victor**
Division of Pediatric Infectious Diseases, UCSD School of Medicine, 9500 Gilman Drive, Mail Code 0687, La Jolla, CA 92093-0687, USA

**Ölschläger, Tobias**
Institut für Molekulare Infectionsbiologie, Röntgenring 11, D-97070 Würzburg, Germany

**Olsnes, Sjur**
Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

**Oram, Diana Marra**
Dept. of Microbiology, Campus Box B-175, Univ. of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262, USA

**Parker, Michael W.**
Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, 9 Princes St., Fitzroy, Victoria 3065, Australia

**Perier, Aurélie**
Département d'Ingénierie et d'Etudes des Protéines (DIEP) bat 152, CEA-Saclay, 91191 Gif sur Yvette Cedex, France

**Pizza, Mariagrazia**
IRIS Research Center, Chiron Vaccines, Via Fiorentina, 1, 53100 Siena, Italy

**Polekhina, Galina**
Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, 9 Princes St., Fitzroy, Victoria 3065, Australia

**Popoff, Michel R.**
CNR Anaerobies et Botulisme, Unite Bacteries anaerobies et Toxines, Institut Pasteur, Paris Cedex 15, Paris F-75724, France

**Poulain, Bernard**
Institut des Neurosciences Cellulaires et Intégrative, CNRS, 5 rue Blaise Pascal, F-67084, Strasbourg Cedex, France

**Prévost, Gilles**
Institut de Bactériologie, 3 rue Koeberlé, Strasbourg F-67000, France

**Proft, Thomas**
Department of Molecular Medicine & Pathology, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Rd., Grafton, Auckland, New Zealand

**Rappuoli, Rino**
IRIS Research Center, Chiron Vaccines, Via Fiorentina, 1, 53100 Siena, Italy

**Rood, Julian I.**
Department of Microbiology, Monash University, Room 131M, Building 27, Clayton, Victoria 3800, Australia

**Rossjohn, Jamie**
Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Faculty of Medicine, Monash University, Clayton, Victoria 3168, Australia

**Rupnik, Maha**
Medical Faculty, University of Maribor, Slomskov trg 15, SI-2000 Maribor, Slovenia

**Salim, Kowthar Y.**
Department of Microbiology, Faculty of Dentistry, University of Toronto, Toronto, Canada

**Salinas, Sara**
Molecular NeuroPathobiology Laboratory, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

**Sandkvist, Maria**
Dept. of Biochemistry, Jerome H. Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA

**Sandvig, Kirsten**
Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

**Schiavo, Giampetro**
Molecular NeuroPathobiology Laboratory, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

**Scortti, Mariela**
Bacterial Molecular Pathogenesis Group, Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, Langford BS40 5DU, UK

**Scott, Maria E.**
Dept of Biochemistry, Jerome H. Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA

**Sears, Cynthia L.**
Johns Hopkins University School of Medicine, 720 Rutland Ave, Ross Building Room 1167, Baltimore, MD 21205, USA

**Sebo, Peter**
Cell and Molecular Microbiology, Institute of Microbiology, Czech Academy of Sciences, Videnska 1083, Prague 142 20, Czech Republic

**Shinoda, Sumio**
Faculty of Science, Okayama University, Okayama 700-8530, Japan

**Simonet, Michel**
Université de Lille II, Institut de Biologie de Lille, 1 rue du Professeur Calmette, Lille Cedex, B.P. 245 -59021, France

**Smith, Leonard A.**
Integrated Toxicology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011, USA

**Stachowiak, Radek**
Bacterial Molecular Pathogenesis Group, Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, Langford BS40 5DU, UK

**Stevens, Dennis L.**
Department of Veterans Affairs, Medical Center, 500 West Fort Street, Boise, ID 83702-4598, USA

**Stiles, Bradley G.**
Integrated Toxicology Division, U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702-5011, USA

**Svennerholm, Ann-Mari**
Dept. Med. Microbiol. Immunol., Göteborg University, Göteborg S-41346, Sweden

**Tang, Julian**
Biota Structural Biology Laboratory, St. Vincent's Institute of Medical Research, 9 Princes St., Fitzroy, Victoria 3065, Australia

**Thelestam, Monica**
Microbiology and Tumor Biology Center, Karolinska Institute Box 280, Stockholm S-171 77, Sweden

**Titball, Richard W.**
Defence Microbiology Division, Chemical & Biological Defence Establishment, Salisbury SP4 0JQ, UK

**Tweten, Rodney K.**
Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA

**Uchiyama, Takehiko**
Department of Microbiology and Immunology, School of Medicine, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 152-8666, Japan

**van der Goot, F. Gisou**
Dept. of Microbiology and Molecular Medicine, University of Geneva, 1 rue Michel Servet, Geneva CH-1211, Switzerland

**Vásquez-Boland, José A.**
Bacterial Molecular Pathogenesis Group, Veterinary Molecular Microbiology Section, Faculty of Medical and Veterinary Sciences, University of Bristol, Langford BS40 5DU, UK

**Wesche, Jørgen**
Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo Norway

**Wilson, Brenda Anne**
Department of Microbiology, University of Illinois at Urbana-Champaign, 601 S. Goodwin Avenue, B103 CLSL, Urbana, IL 61801, USA

**Wu, Shaoguang**
Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

# Preface to Third Edition

An increasingly rapid expansion of our knowledge of the multifaceted aspects of bacterial protein toxins since the end of the 20th century necessitated the publication of this third edition of *The Comprehensive Sourcebook of Bacterial Protein Toxins.*

Among the most important achievement and discovery of the past six years was the explosion of new information in many domains of toxinology, illustrated by the discovery of more than 50 novel toxins (many of them identified through genome screening), extensive bacterial genome mapping, the determination of the nucleotide sequence of encoding genes and corresponding amino acid sequences, genetic regulation of toxin expression, genomic pathogenicity islands, identification of many toxin receptors at the surface of eucaryotic cells, toxin-induced modulation of various components, and effectors of intracellular signal transduction pathways and apoptotic events. Establishment of the three-dimensional crystal structure of more than 20 toxins during the same period allowed deeper knowledge of structure-activity relationships and provided a framework for understanding how toxins recognize receptors, penetrate membranes, and interact with and modify intracellular substrates. Moreover, the study of the structure of newly identified toxins demonstrated that toxins, which modify the cellular signaling pathways, possess the same active sites (functional mimicry) as those of corresponding cell proteins but with a different folding conformation. These new findings allow a better understanding of the mechanism of action of bacterial toxins at the subcellular level. In addition, bacterial toxins are extremely specific and exquisite tools to unravel physiological cell processes.

Recent progress also contributed to a better definition of major issues, namely toxin transcytosis and trafficking in eucaryotic target cells, mechanisms of action at the cellular and molecular levels, genetic and molecular mechanisms of toxin involvement in the virulence and pathogenicity of the relevant bacteria in many human diseases (cholera, anthrax, whooping cough, tetanus, botulism, diptheria, clostridial gas gangrenes, severe diseases caused by superantigenic toxins, *Helicobacter pylori*-associated peptic ulcers and carcinomas, food poisons, etc.). In this respect, the targeting of immune system cells by various toxins led to a better evaluation of both their beneficial effects (for example as immunomodulators in the case of cholera toxin B subunit) and pathophysiolgical effects in certain diseases in connection with the immune system. Finally, the past years witnessed considerable progress on toxin applications in vaccinology, tumor therapy, and new approaches in the treatment of various diseases. Whether some wild-type toxins can be directly used as therapeutic agents, protein engineering permits us to model more specific and efficient molecules or molecules with a novel activity, or to target a restricted subset of cell population. Novel recombinant toxins are already proposed in the treatment of some diseases, as well as new vaccines. One should also not forget the emergence of certain bacterial toxins as biological weapons (era of bioterrorism threats). This issue is considered in chapters dedicated to the relevant toxins (anthrax toxins, botulinal neurotoxins, the Shiga-like plant toxin, ricin) and is also discussed in a specific chapter.

The third edition of the *Sourcebook* is a genuine, timely production comprising 62 chapters (compared to 40 and 20 of the 1999 and 1991 editions, respectively) that are organized into five sections. They are written by a panel of 137 international experts (senior authors and coauthors), who have significantly contributed to the progress within their featured disciplines. About 55% of all contributors are new specialists who did not contribute to the preceding editions of the *Sourcebook*. Authors are from Australia, Belgium, Canada, the Czech Republic, France, Germany, Italy, Japan, New Zealand, Norway, Sweden, Switzerland, the United Kingdom, and the U.S.A.

As editors, we are keen to provide the reader with a sound, authoritative, and interdisciplinary review highlighting the major advances in the field of bacterial protein toxins. Whenever possible, particular emphasis was placed on toxin involvement in the pathogenesis of the diseases caused by the relevant bacteria. At the same time, 14 totally new chapters were created to cover the historical and general aspects of toxinology.

We hope the *Sourcebook* will appeal to a wide readership, including microbiologists and particularly toxinologists, biochemists, cell biologists, clinicians, and medical students who wish to have a better understanding of bacterial toxins in relation to infectious diseases.

We are heavily indebted to all contributors who skillfully documented developments at the cutting edge of their disciplines. Their painstaking efforts are deeply appreciated.

We wish to express our deep thanks to Tessa Picknett, Senior Publisher at Academic Press, Elsevier and her coworkers, Bryony Lott, Victoria Lebedeva (London), and Jeff Freeland (Burlington, USA) for their encouragement and invaluable help through the preparation of this volume.

*Joseph E. Alouf and Michel R. Popoff*

# Preface to Second Edition

It is now eight years since the publication of the first edition of *The Comprehensive Sourcebook of Bacterial Protein Toxins*, and during that period many previously well-recognized toxins now constitute the prototypes of toxin families which share common mechanisms and, most probably, common evolutionary origins (e.g. the RTX toxins, STs and LTs of enterobacteria). Of great significance in this context was the recent recognition of pathogenicity islands which are responsible for the mobility and horizontal spread of toxins and other virulence genes between closely related species. Further remarkable progress has been made in the definition of molecular mechanisms of a wide range of toxins, with increasing numbers having enzymic mechanisms revealed, including ADP-ribosylation and glycosylation of novel targets (e.g. small Ras G-proteins). Among the most exciting discoveries of the 1990s, simply because it explained the potency of botulinum and tetanus toxins which have challenged toxinologists for over 100 years, was the demonstration that they consist of Zn-proteinases of exquisite specificity, cleaving proteins associated with vesicle fusion at presynaptic membranes. We now have the three-dimensional structure of botulinum toxin, which should yield further detailed information on the functional domains of these fascinating molecules.

The host immune system is not only the primary defence against colonization, and sometimes invasion, by toxigenic bacteria but it also constitutes the major target for a growing repertoire of bacterial toxins which can act either directly by cytoxicity towards immune effector cells (e.g. the leucocidins of *Staphylococci* and the leucotoxins of *Pasteurellae*) or by more subtle routes involving deregulation of cytokine production (e.g. the superantigenic toxins of *Staphylococci* and *Streptococci*).

Genome analysis promises to identify many more toxins over the next decade and should provide rich pastures for functional analysis in the next century. These analyses undoubtedly offer new opportunities for use as tools to dissect cellular processes as well as novel potential therapeutic agents.

A volume such as the *Sourcebook*, although attempting to cover the major groups of toxins at a relatively detailed level, inevitably has some gaps, and if these occur in your favorite area of research, then we can only apologize. The content of the book always reflects a compromise between what the editors would like and what they are able to include, yet still meet the deadlines set for the production of the book. We are greatly indebted to all the contributors, especially to those who met the first submission deadline. We wish to record our thanks to Tessa Picknett at Academic Press, who initiated the second edition, and to Lilian Leung who saw the project through to a successful conclusion with persistence and good humour. We also thank Mrs. Patricia Paul for invaluable secretarial work in compiling chapters and correspondence with authors.

*Joseph E. Alouf and John H. Freer*

# Preface to First Edition

Great strides have been made in the depth of our understanding of the structure and mechanisms of action of bacterial toxins over the last decade. The current pace of this advance in knowledge is particularly impressive, and results largely from the power that gene manipulation techniques have offered in experimental biology.

Recent research achievements in the field of bacterial toxins, which consist of about 240 protein toxins as well as a relatively small number of non-protein toxins, reflect the extensive and productive blending of disciplines such as molecular genetics, protein chemistry and crystallography, immunology, neurobiology, pharmacology and biophysics. Furthermore, the exciting developments in many areas of cell biology, and particularly in membrane-associated mechanisms relating to signalling and communication, export and import of proteins and to cytoskeletal functions, have been facilitated because critical steps in these processes constitute the targets for bacterial toxins. Thus, we have toxins available which can be used to probe many fundamental aspects of eukaryotic cell biology.

Disruption of these same central cellular processes *in vivo* can also be the critical event in the pathogenesis of infectious diseases for man or domestic animals. Many such infectious diseases have major social or economic impacts on man, and such considerations have quickened the pace of the search for therapeutic agents. Currently, a number of physically inactivated bacterial or hybrid engineered toxoids are used as immunogens in vaccination programmes, and there is a major international effort to develop new and more effective vaccines based on our deeper understanding of the molecular events in pathogenesis and the host response to infection.

Since the publication of the excellent multi-volume treatise on *Bacterial Toxins* edited by S. Ajl, S. Kadis and T. Montie (Academic Press) in the early 1970s, most of the books published in the past twenty years have covered the subject by presenting individual toxins or groups of toxins in separate chapters. This is not the main approach followed in this book. Our aim is not to give an exhaustive review of the wide spectrum of the protein toxin repertoire but rather to give an "in depth" critical review of the original and the newly expanding body of information accumulated during the past decade or so. The multifaceted aspects of toxin research and the multidisciplinary approaches adopted suggested to us that "state of the art" toxin research might best be presented by putting together in several chapters the common structural and/or functional aspects of toxin "families". Other chapters highlight the various physiological or genetic mechanisms regulating toxin expression and the therapeutic or vaccine applications of genetically engineered toxins.

The 22 chapters of this book have been written by 44 internationally known specialists who have significantly contributed to the progress in the domains covered. It is hoped that this book will appeal to a wide readership, including microbiologists, biochemists, cell biologists and physicians. Also, we hope it will arouse the interest of students and scientists in other disciplines who see the power of these fascinating biological agents, either as exquisitely specific probes of cellular processes or as extremely potent agents of infectious disease.

Finally, we would like to thank all the authors for their contributions, and particularly to those who delivered their manuscripts by the first deadline. We also express our appreciation to the editorial staff at Academic Press for their help and patience throughout the preparation of this book.

*J.E. Alouf and J.H. Freer*

# Introduction

Since the second edition of *The Comprehensive Sourcebook of Bacterial Protein Toxins* was published in 1999, major world events have impacted research on infectious agents, including pathogenic bacteria and their toxins. Most notably, the anthrax-tainted letters delivered via the United States Postal Service in the fall of 2001 sent a shock wave around the world. Although the letters produced only limited morbidity and mortality (22 cases of anthrax, 5 of which were fatal), they caused massive and costly disruption of the United States postal and legislative systems, and exacerbated the terror resulting from the attacks with airliners on the World Trade Center and the Pentagon. As a consequence, there has been a general widespread recognition of the threats from infectious diseases and a major reorientation in priorities for funding biological research. This is exemplified by the establishment of a set of Regional Centers of Excellence for Biodefense and Emerging Infectious Diseases Research across the United States under National Institutes of Health sponsorship. The number of laboratories performing research on the causative agents of anthrax, smallpox, and other diseases on the A, B, and C "select lists" has increased dramatically, of course.

In facing the threats of bioterrorism, emerging infectious diseases, and ancillary problems, such as antibiotic resistance, the international community of scientists is fortunate in being able to draw on a rapidly expanding body of knowledge about pathogenesis. The past several decades have seen a major rise in interest in infectious disease research, due perhaps more than anything else to the development and convergence of methods directed primarily towards answering questions in basic science. The protein toxins produced by bacteria represent an interesting focus to illustrate this point. These toxins have always been of interest from the biological and medical communities, but in recent decades have also attracted biochemists and biophysicists expert in the array of tools developed to examine protein structure and function—x-ray crystallography, fluorescence spectroscopy, directed mutagenesis, mass spectrometry, and so forth. As a result, we now know the atomic level structures of a great many of these proteins, and are rapidly coming to understand their conformational dynamics and the details of their molecular interactions with host molecules (receptors, substrates, etc.). Cell biologists have brought to the table a complementary set of tools—confocal microscopy and fluorescent markers, for example, to track the attachment, entry, and trafficking of toxins. Along the way, the neurobiologists have become interested because some toxins form pores, resembling ion channels, in membranes. And the details of the molecular structures and of molecular and cellular function present questions of mode of action at tissue and organismal levels in a new, more precisely defined form to the physiologists and pathologists. As all of this is occurring, questions pertaining to the array of toxins encoded in bacterial genomes, or in mobile genetic elements, and how their production is controlled, and how they are secreted from the bacteria, are being solved through the application of molecular genetics. This grand evolving body of knowledge presents the scientist seeking to develop new countermeasures to biological threat agents, be they microbes or their toxins, with unprecedented opportunities for dissecting their own individual systems.

As in the previous editions, the third edition of *The Comprehensive Sourcebook of Bacterial Protein Toxins* serves to compile the latest and most relevant information to inform, stimulate, and excite, the researcher, the teacher, and the student.

*R. John Collier*

# INTRODUCTORY SECTION

CHAPTER

# 1

# A 116-year story of bacterial protein toxins (1888–2004): from "diphtheritic poison" to molecular toxinology

*Joseph E. Alouf*

*To the physiologist the poison becomes an instrument which dissociates and analyzes the most delicate phenomenon of living structures and by attending carefully to their mechanism in causing death, he can learn indirectly much about the physiological processes of life.*

*Claude Bernard*, La Science Experimentales, *Paris, 1878*

## INTRODUCTION

The concept that pathogenic bacteria might elicit their harmful effects in humans and animals by means of poisons elaborated by these microorganisms in the infected host is almost as old as the notion of pathogenic bacteria itself (William Edward van Heyningen 1950, 1970). The heuristic impact of the concept of bacterial poisons was considerable after the discovery of bacteria and their relation to disease. It was undoubtedly influenced by the knowledge that other biological organisms do produce poisons such as animal venoms and plant alkaloids. Indeed, the idea that the harm caused by infectious disease might be due to microbial poisons was entertained long ago even before the germ theory of disease was established. Both endotoxins and exotoxins were foreseen in 1713 by Vallisnieri, who suggested on one page of his *Riposta* that the "little worms of the most atrocious pests are of themselves of a poisonous nature" (van Heyningen, 1955).

The first attempts to demonstrate experimentally the production of bacterial poisons were undertaken by Edwin Klebs (1834–1913) for staphylococci, Robert Koch (1843–1910) for *Vibrio cholereae*, and Friedrich Loeffler (1852–1915) for diphtheria bacillus, respectively. These attempts failed for methodological reasons that are now understandable.

Klebs (1872) suggested that chemical substances named "sepsins" were responsible for the lesions caused by staphylococci, but no evidence for the existence of such substances was found. The first experimental demonstrations of staphylococcal toxin(s) were reported later by von Leber (1888), De Christmas (1888), van de Velde (1894), and other microbiologists by the early 1900s (see Arbuthnott, 1970).

As concerns cholera, Koch (1884) expressed the view that the disease was a toxicosis, since the causative organism proliferated in the gut without appearing to invade or damage this organ or any of the neighboring tissues. Parenteral injection of *V. cholereae* filtrates did not produce any toxic effects in experimental animals, and the idea of an extracellular poison was abandoned. However, 75 years later, Koch's hypothesis was confirmed when two Indian researchers, De (1959) and Dutta *et al*. (1959) working independently of one another, showed that cell-free preparations from *V. cholerae* caused relevant symptomatology in animal models (adult rabbit ligated loop and infant rabbit, respectively). In 1969, the putative toxin was purified and biochemically characterized by

Finkelstein and Lo Spalluto (1969). Fourteen years later, the cloning and nucleotide sequence of the toxin was determined by Mekalanos *et al*. (1983). Cholera toxin, an 84-kDa oligomeric protein; is the prototype of a wide family of biochemically, immunologically, and pharmacologically related toxins found in human and porcine *E. coli* strains, non 01 *V. cholerae*, *V. mimicus*, *Aeromonas hydrophila*, *Campylobacter jejuni*, *Salmonella enterica serovar typhi*, and *typhimurium* and *Plesiomonas shigelloides* (Sears and Kaper, 1994 and Chapter 19 of this volume).

The same year of Koch's hypothesis, Loeffler (1884) suggested that a soluble poison was the causative agent of diphtheria. This author who had discovered diphtheria bacillus reported that in animals that died after experimental infection, the microorganism remained at the seat of inoculation and was totally absent from the internal organs. Nevertheless, these organs were profoundly damaged ("hemorrhagic edema, effusion into the pleural cavities, catarrhal inflammation of the kidneys, and deep reddening of the suprarenal capsules"). Loeffler concluded that "this clearly indicated that a poison at the seat of inoculation must have circulated in the blood." However the putative poison was not found.

## REPERTOIRE OF BACTERIAL PROTEIN AND PEPTIDE TOXINS (1888–2004)

This repertoire comprises so far 339 members including natural variants, serotypes, isoforms, and allelic forms, namely 160 (47%) from Gram-positive bacteria and 179 (52%) from Gram-negative bacteria. Most of them (85%) are extracellular or cell-associated and the rest (15%) are intracellular. As established in the past 30 years, many toxins were found to possess a variety of enzymatic activities (Table 1.1).

## DISCOVERY OF THE FIRST BACTERIAL PROTEIN TOXINS: DIPHTHERIA, TETANUS, AND BOTULINAL TOXINS (1888, 1890, 1896)

### Diphtheria toxin

In 1888, fortune smiled on Emile Roux (1853–1933) and Alexandre Yersin (1863–1943). At Pasteur Institute, these researchers discovered the first bacterial toxin, namely diphtheria toxin (Roux and Yersin, 1889), six months after the creation by Louis Pasteur (1822–1895) of this "Temple of Science." The "diphtherial poison" was detected in sterile filtrates of diphtheria bacillus (*Corynebacterium diphtheriae*). The filtrates or the material isolated by cadmium chloride precipitation from old alkaline cultures injected into guinea pigs, rabbits, and pigeons mimicked the symptoms and type of death produced by infection with living organisms. Moreover, Roux and Yersin demonstrated that the urine of children taken shortly before death from diphtheria may contain sufficient toxic material to kill guinea pigs with symptoms similar to those elicited by culture filtrates. The earlier prediction of Loeffler was thus verified. For the first time, the mechanism of pathogenicity of a microorganism in humans became clarified and could be explained in terms of a soluble toxic substance released by bacteria named for the first time a *toxin* by the authors (from Greek: τοξιχον, poison) (van Heyningen, 1970). However, according to Carl Lamanna (1990), the term *toxin* was first used in 1886 by E. Ray Lankester in *Science* to name "poisons for animals produced by pathogenic bacteria." Very likely, Roux and Yersin were not aware of Lankester's article.

#### *Rational design and serendipity*

The seminal discovery of the "diphtheritic poison" was the fruit of both rational design and serendipity. The culture medium used for bacterial growth was prepared with Paris city tap water, which was known to contain high amounts of calcium. Autoclaving led to the precipitation of calcium phosphate and thereby the lowering of iron concentration in the medium, which empirically favored diphtheria toxinogenesis, as reported ca. 40 years later by Locke and Main (1931), Pope (1932), and Pappenheimer and Johnson (1936). Toxin production was maximal under iron starvation conditions and was severely inhibited under high–iron growth conditions. It is interesting to report how Pappenheimer discovered the "iron effect." He found that cultures growing on a particular medium would produce toxin one day and fail to produce it on the "same" medium the next day. Then his laboratory received a supply of new hard-glass bottles, and in these, toxin was produced without fail. When small amounts of crushed glass from the soft-glass bottles were introduced in the new bottles, toxin production went down. It was found that the inhibition of toxinogenesis was due to the high iron content of the soft glass.

The discovery of the iron effect in the 1930s was a very important event in the history of bacterial toxins (Alouf and Raynaud, 1960). It paved the way for a number of major achievements in toxinology over the past 50 years.

Freeman (1951) discovered that diphtheria toxin is encoded by a bacteriophage gene *tox*+ called β (see

**TABLE 1.1** Toxins exhibiting enzyme activity

**1. ADP-RIBOSYLATING TOXINS**
Transferase and NADase activity
Ref. Han and Tainer (2002); Masignani *et al*. (2004) and Chapter 12 of this volume

**2. PHOSPHOLIPASES**
Cytolytic phospholipases C: *C. perfringens* zinc-dependent α-toxin, *C. sordellii* γ-toxin, *C. novyi* β and γ-toxins, *C. heamolyticus*, *P. aeruginosa*, *P. aureofaciens*, *A. hydrophila, B. cereus, Acinetobacter calcoaceticus* haemolysins
*Vibrio damsela* cytolytic phospholipase D
*Rickettsia prowazekii* haemolytic phospholipase A
*S. aureus* β-toxin (haemolysin, sphingomyelinase)
*Corynebacterium ovis* lethal toxin (phospholipase D)
*Yersinia pestis* murine toxin (phospholipase D)
Ref: Hatheway, C.L (1990); Songer (1997); Jepson and Titball (2000); Hinnebusch *et al*. (2000).

**3. ADENYLATE CYCLASES**
*Bordetella pertussis*, *B.parapertussis* and *B. bronchiseptica*, *Bacillus anthracis* bifactorial oedema toxin, *Pseudomonas aeruginosa* exotoxin Y
Ref. Confer and Eaton (1982); Ladant and Ullmann (1999); Hewlett and Gray (2000); Leppla (1988); Lory *et al*. (2004), Yahr *et al*. (1998)

**4. METALLOPROTEASES**
*Tetanus* and *botulinum* A, B, C, D, E, F, G (light chain) zinc-dependent neurotoxins
*Bacillus anthracis* bifactorial lethal toxin, *Bacteroides fragilis* zinc-dependent enterotoxins
Ref. Herreros *et al*. (1999); Rossetto *et al*. (2001); Lalli *et al*. (2003); Franco *et al*. (1997); Pannifer *et al*. (2001); Vitale *et al*. (2000)

**5. RNA *N* -GLYCOSIDASES**
*S. dysenteriae* shiga toxin, *E. coli* shiga-like toxins (verotoxins: Stx 1, 1c, 2, 2c, 2d, 2e) and similar toxins from *Aeromonas hydrophila, A. cassia, Enterobacter cloacae, Citrobacter freundii;* ricin and other plant toxins
Ref. Endo (1988); Tesh and O'Brien (1991); O'Loughlin and Robins-Brown (2001)

**6. GLUCOSYL TRANSFERASES**
*C. difficile* A, B toxins, *C. sordellii* and *C. novyi* lethal α-toxin
Ref. Aktories (2003); von Eichel-Streiber *et al*. (1996); Selzer *et al*. (1996)

**7. DEAMIDASE ACTIVITY**
*E. coli* cytotoxic necrotizing factor-1
Ref. Flatau *et al*. (1997); Schmidt *et al*. (1997)

**8. PROTEASE ACTIVITY**
*S. aureus* epidermolytic toxins (exfoliatins) serotypes A, B, C, D; *S. hyicus* exfoliatins at least six serotypes; SHETA, SHETB, Exha, Exhb, Exhc, Exhd
Enteroaggregative *E. coli* pet toxin; *S. pyogenes* cysteine proteinase
Ref. Melish *et al*. (1974); Yamaguchi *et al*., (2002); Ladhani *et al*. (2002); Hanakawa (2004); Ahrens and Andresen (2004); Villaseca *et al*. 2000; Gerlach *et al*. (1994) and Chapter 56 of this volume.

**9. DEOXYRIBONUCLEASE ACTIVITY**
Cytolethal distending toxins (18 members at least from various Gram-negative bacteria)
Ref. Johnson and Lior (1988a); Pickett and Whitehouse (1999); Dreyfus (2003); Thelestam and Frizan (2004) and Chapter 23 of this volume.

Groman, 1953; Barksdale and Arden, 1974). Then Murphy *et al*. (1978) and Welkos and Holmes (1981) demonstrated that the regulation of toxin expression by iron occurred at the level of *tox* gene transcription under the control of a chromosomal *dtxR* gene encoding a diphtheria toxin repressor (see White *et al*., 1998; Goranson-Siekierke and Holmes, 1999; and Chapter 13 of this volume).

Since the pioneering works on the involvement of iron in the regulation of bacterial toxingenesis, a great number of investigations have been undertaken for other toxins or virulence factors whose synthesis is negatively regulated by iron. This is the case of *Shigella dysenteriae* shiga toxin, *Escherichia coli* Shiga-like toxin-1, and hemolysin, *Vibrio cholerae* hemolysin, *Pseudomonas aeruginosa* exotoxin A, *Clostridium tetani* tetanus toxin, *Helicobacter pylori* VacA cytotoxin, *Plesiomonas shigelloides* CHO cell elongation factor (Hantke *et al*., 2001; Payne, 2003).

### *"What is the nature of the diphtheric poison? Is it an alkaloid or a diastase?"*

Roux and Yersin (1888) addressed these questions and suggested that because of its heat lability, the toxin resembled an enzyme ("diastase") more than an alkaloid. However, eighty years intervened before it was finally proved independently in two laboratories (Honjo *et al*., 1968; and Gill *et al*., 1969) that diphtheria toxin is, in fact, a highly active enzyme of a novel and unique type (Collier, 1975). The toxin inhibits eucaryotic protein synthesis by catalyzing the covalent transfer of the ADP-ribose portion of nicotinamide (NAD) to elongation factor 2, which in the case of diphtheria toxin leads to target cell death. Interestingly, prior to this very important achievement the pioneering experiments of Lennox and Kaplan (1957) and Placido-Souza and Evans (1957) showed that the toxin was lethal for cells in culture from sensitive animals. Then, the elegant experiments of Strauss and Hendee (1959) on HeLa cells provided the first indication that cell death resulted from the inhibition of protein synthesis by diphtheria toxin (see Collier, 1977 for a comprehensive review of this issue).

### *Diphtheria toxin: eponymous archetype of a vast class of bacterial toxins and virulence factors*

The pioneering discovery of the ADP-ribosyltransferase activity of diphtheria toxin opened a new era in

our knowledge of bacterial toxins, their mode of action, and their role in the pathogenesis of various infectious diseases (Barbieri and Burnes, 2003). Indeed, this toxin is the first member and the prototype of a vast family of conserved ADP-ribosyltransferases (ADPRTs) from various bacterial pathogens that display a variety of pharmacologic, physiologic, and toxic activities toward humans and (or) animals. These effectors constitute an immense potential for modern biomedical research.

## Discovery of clostridial neurotoxins

Two other major toxins were to follow soon after the discovery of diphtheria toxin: tetanus toxin in 1890 and botulinum toxin in 1896, produced by *Clostridium tetani* and *Clostridium botulinum*, respectively.

### *Tetanus toxin*

Tetanus was recognized since ancient times and described by Hippocrates who first reported the symptoms of a sailor affected by a syndrome characterized by hypercontraction of the skeletal muscles. He termed such a spastic paralysis "tetanus" (from the Greek word τετανυσ, tension).

A "clinical" description of the disease was remarkably illustrated by the Scottish surgeon Sir Charles Bell in his book *The Anatomy and Philosophy of Expression* published in 1832. In 1884, Carle and Rattone showed that tetanus could be transmitted from a human suffering from the disease to a rabbit injected with material from the wound. Implantation of soil samples into mice or rabbits caused symptoms of tetanus. In smears from the wound, *drumstick* forms (*clostridium* in Latin) of *C. tetani* were first observed by Nicolaier (1884), who noted that the microorganism was not distributed in the whole organism, but confined to the wound of entry. Simpson had already observed in 1856 that the symptoms of tetanus were similar (as confirmed later) to those of strychnine poisoning and in 1885 Nicolaier suggested that the microorganism brought about its pathological effects by producing a strychnine-like poison. In 1889, Shibasaburo Kitasato (1852–1931) obtained a pure culture of the bacterium in Robert Koch's laboratory in Berlin.

The toxin was discovered in 1890 in culture supernatents in Denmark by Knud Faber and in Italy by Tizzoni and Cattani (1890). Faber justified Nicholaier's suggestion by showing that it was possible to reproduce the spastic symptoms of tetanus in experimental animals by injecting them with sterile filtrates of cultures of the bacillus (van Heyningen and Mellanby, 1971). The toxin producing these effects was also named *tetanospasmin* (for other references see Rossetto *et al*., 2001; Lalli *et al*., 2003). The toxin was shown by Bruschettini (1897) to move retroaxonally and to act at the spinal-cord level. Active immunization with formaldehyde-treated tetanus toxin was undertaken by Ramon and Descombey (1925).

*Tetanus and diphtheria antitoxins: the birth of serotherapy and experimental immunology*

In December of the same year, Behring and Kitasato (1890) in Berlin rendered mice and rabbits resistant to the toxic effects of tetanus and diphtheria toxins by inoculating the animals with small doses of toxin preparations attenuated by "Gram liquor" (iodine). The sera of the "immunized" animals not only specifically neutralized the toxins, but still did so when transferred into the bodies of "native" animals challenged with the native toxins. This experiment revealed for the first time the production of neutralizing "Antikörper" (antibodies) and led to the development of serotherapy and to the birth of experimental immunology. In 1884, Emile Roux announced the large-scale production of horse immune sera (antitoxins) for the serotherapy of diphtheria (Roux and Martin, 1884). This was also the case for the preparation of large amounts of antitetanus antitoxins by Behring (1892).

*Diphtheria and tetanus toxoids*

Another epochal achievement was the independent development of toxoids by Glenny and Sudmersen (1921) in the United Kingdom and Gaston Ramon (1923; Ramon and Descombey, 1925) in France for use in human vaccination. Since then mass immunization with diphtheria and tetanus toxoids has led to a remarkable decrease of the two diseases in developed countries. Unfortunately, around 500,000 annual cases of tetanus (particularly neonatal) are still reported in countries of the Third World for lack of vaccination.

### *Botulinum toxins*

Botulism (*sausage poisoning* in Latin) is the term given to an acute food poisoning caused by the ingestion of spoiled sausages (*botulus)* that has been known as long as 1,000 years ago. The Byzantine Emperor Leo VI (886–911 A.D.) forbade the preparation and eating of blood sausages.

Centuries later (1793) in Württemberg, an outbreak affected 13 persons, six of whom died. The outbreak was attributed to blood sausage consumption; thereby the preparation of this food came under strict government regulation.

In 1820, Justinus Kerner, a poet turned physician and medical officer for the Duchy of Württemberg, reported 200 cases of sausage poisoning.

The etiology of botulism was elucidated in 1895 by the Belgian microbiologist van Ermengen of the University of Ghent in his investigation of a tragic outbreak of botulism in Ellezelles (Belgium) that involved 50 cases among members of a musical society who partook of a meal at a funeral. Three of them died and at least 10 were seriously ill (van Ermengen, 1897).

The outbreak was caused by a salt-cured uncooked ham. Portions of macerated ham fed to mice, guinea pigs, and monkeys caused paralytic signs of the illness observed in the patients and subsequently resulted in death. Filtered extracts of the ham had the same effects as the macerated ham.

Van Ermengem consistently found an anaerobic sporulating bacillus in cultures of the ham, as well as in a culture of the spleen from one of the deceased victims. The organism was named *Bacillus botulinus* and thereafter, *C. botulinum*. Culture filtrates had the same effect as did ham macerates (Sakagushi, 1986; Rossetto *et al*., 2001).

*Discovery of different types of botulinum toxins*

In 1904, 11 persons died from eating wax-bean salad in Darmstadt (Germany). The antitoxin serum prepared against the Ellezelles strain did not neutralize the toxin from the Darmstadt strain and reciprocally. Thus, the toxins from the two strains were immunologically distinct (Leuchs, 1910) and were later classified into types A and B (Meyer and Gunnison; 1929 in Sakaguchi's chapter). Type B corresponded to the Ellezelles strain. Types C and D were characterized by Bengston (1922) and Theiler (1927). Types E and F were identified by Gunnison (1936) and Dolman and Murakami (1961), respectively. Type G was isolated from a soil sample collected in Argentina (Giménez and Sicarelli, 1970). (See Sakagushi, 1986; Popoff and Marvaud, 1999; Herreros *et al*., 1999 for references.)

*Recent developments of the research on clostridial neurotoxins*

Great advances in our understanding of the molecular mechanisms of action of these neurotoxins have been made in the past 15 years. Both toxins are structurally related 150-kDa zinc-dependent metalloproteases consisting of three domains, endowed with different functions: neurospecific binding, membrane translocation, and specific proteolysis of three key components of the neuroexocytosis apparatus. After binding to the presynaptic membrane of motoneurons, tetanus neurotoxin (TeNT) is internalized and transported retroaxonally to the spinal cord, where it blocks neurotransmitter release from spinal inhibitory interneurons. In contrast, the seven botulinum neurotoxins (BoNT) act at the periphery and inhibit acetylcholine release from peripheral cholinergic nerve terminals. TeNT and BoNT-B, -D, -F, and -G cleave specifically at single but different peptide bonds, VAMP/synaptobrevin, a membrane protein of small synaptic vesicles. BoNT types -A, -C, and -E cleave SNAP-25 at different sites within the COOH-terminus, whereas BoNT-C also cleaves syntaxin. BoNTs are increasingly used in medicine for the treatment of human diseases characterized by hyperfunction of cholinergic terminals (see Rossetto *et al*., 2001; and Chapters 19 and 20 of this volume). The genes encoding botulinum and tetanus neurotoxins have been widely investigated during the past 25 years as recently reviewed by Raffestin *et al*. (2004). BoNT -A,-B, -E, and -F are encoded by chromosomal genes; BoNT -C and -D are encoded by bacteriophages, while BoNT-G and tetanus neurotoxin genes are localized on large plasmids in *C. argentinense* and *C. tetani*, respectively. Moreover, the regulatory genes involved in the production of clostridial neurotoxins have been recently identified (Raffestin *et al*., 2004). The three-dimensional crystal structure of BoNT-A, -B, and -E and tetanus toxin was determined by the end of the 1990s (Table 1.2).

## TOXIN RESEARCH BETWEEN 1900 AND 1975

Since the discovery of the first three major bacterial protein toxins, about 100 new protein toxins produced by a number of Gram-positive and to a lesser extent from Gram-negative bacteria were identified during the first half of the twentieth century. Much effort was particularly focused on anaerobic clostridial species and their toxins as a result of the experience gained during World War I gas gangrene on the battle field. Research on other toxins during the 1900–1965 period was also developed on toxinogenic anaerobes and certain aerobic bacterial pathogens, such as staphylococci, streptococci, *Bacillus anthracis*, and on various membrane-damaging toxins from both Gram-positive and Gram-negative bacteria.

### *Clostridia*

The genus *Clostridium* encompasses over 80 species of Gram-positive, anaerobic spore-forming bacteria. Fifteen account for a wide range of diseases in humans and animals by virtue of their capacity to produce highly potent extracellular protein toxins that are responsible for the pathogenicity of the microorganisms. The toxinogenic species listed below were identified over a period of about 100 years. To date, 60 toxins have been characterized (Hatheway, 1990 and Table 1.3).

**TABLE 1.2** Three-dimensional structure of crystallized toxins established to date (2004)

1. *P. aeruginosa* exotoxin A (1986). Allured,V.S. *et al. Proc. Nat. Acad. Sci*. **83**, 1320
2. *E. coli* LT-1 toxin (1991). Sixma, T.K. *et al. Nature*, **351**, 371
3. *Bacillus thuringiensis* δ-toxin (1991). Li, J. *et al. Nature*, **353**, 748
4. Oligomer B of *E. coli* shiga-like toxin (1991). Stein, P.E. *et al. Nature*, **355**, 748
5. Diphtheria toxin (1992). Choe, S. *et al. Nature*, **357**, 216
6. *S. aureus* enterotoxin B (1992). Swaminathan, S. *et al. Nature*, **359**, 801
7. *S. aureus* toxic-shock syndrome toxin-1 (TSST-1). (1993, 1994) Prasad , G.S. *et al. Biochemistry*, **32**, 13761; Acharya, K.R. *et al. Nature*, **367**, 94
   TSST-1 -MHC class II complex (1994). Kim, J. *et al. Science* **266**, 1870
8. *Aeromonas hydrophila* proaerolysin (1994). Parker, M.W. *et al. Nature*, **367**, 292
9. *Pertussis* toxin (1994). Stein, P.E. *et al. Structure* **2**, 45
10. *Shigella dysenteriae* toxin (1994). Fraser, J. *et al. Nature Struct. Biol*. **1**, 59
11. Oligomer B of the choleragenoid form of cholera toxin (1995). Zhang, R.G. *et al. J. Mol. Biol*. **251**, 550
12. Cholera toxin (holotoxin) (1995). Zhang, R.G. *et al. J. Mol. Biol*. **251**, 563
13. *S. aureus* enterotoxin C1 (1995). Hoffmann, M.L. *et al. Infec. Immun*. **62**, 3396
14. *S. aureus* enterotoxin C2 (1995). Papageorgiou, A.C. *et al*. Structure *3*, 769; Kumuran *et al*. (2001) Acta Crystallogr. *D Biol. Crystallogr*. **57**, 1270
15. *S. aureus* enterotoxin A (1995,1996). Schad, E.M. *et al. EMBO J*. **14**, 3292
    Sundström, M. *et al. J. Biol. Chem*. **271**, 32212
16. *S. aureus* enterotoxin C3 (1996). Fields, B.A. *et al. Nature* **384**, 188
17. *S. aureus* enterotoxin D (1996). Sundström, M. *et al. EMBO J*. **15**, 6832
18. *S. aureus* α-toxin (1996). Song, L. *et al. Science* **274**, 1859
19. *S. aureus* exfoliative toxin A (1997. Vath, G.M. ***et al***. Biochem. *36*, 1559; Cavarelli *et al. Structure* **5**, 813
20. Anthrax toxin P component (protective antigen) (1997). Petosa, C *et al. Nature*, **385**, 833
21. Hc fragment of tetanus neurotoxin (1997, 2001). Umland, T.C. *et al. Nature Struct. Biol*. **4**, 10, 788; Fotinou, C . *et al. J. Biol. Chem*. **276**, 32274
22. *S. pyogenes* erythrogenic (pyrogenic) exotoxin C. (1997). Roussel, A. *et al. Nature Struct. Biol*. **4**, 635
23. Nucleotide–free diphtheria toxin (1997). Bell, C.E. and Eisenberg, D. *Biochemistry* **36**, 481
24. *S. aureus* enterotoxin B (1998). Papageorgiou, A.C. *et al. J. Mol. Biol*. **277**, 61
25. Botulinum neurotoxin type A (1998). Lacy, D.B. ***et al***., *Nature Structur. Biol*. **5**, 898
26. *S. pyogenes* erythrogenic (pyrogenic) exotoxin A (1999, 2001). Papageorgiou, A.C. *et al. EMBO J*. **18**, 9; Baker, M. *et al. Prot. Sci*. **10**, 1268
27. Streptococcal superantigen (SSA) (1999). Sundberg, E.J. and Jardetzky, T.S. *Nature Struct. Biol*. **6**, 123
28. *S. aureus leucocidin* LukF (1999). Olson, R. *et al; Nature Struct. Biol*. **6**, 134
29. *S. aureus leucocidin* LukF-PV (1999). Pedelacq, J.D. *et al. Structure*, **7**, 277
30. Streptococcal pyrogenic exotoxin H (SPE H) and SMEZ 2 (2000). Arcus, V.L. *et al. J. Mol. Biol*. **299**, 157
31. *S. aureus* enterotoxin H (2000). Hakansson *et al. J. Mol. Biol*. **302**, 527
32. Botulinum neurotoxin type B (2000). Swaminathan, S. and Eswaramoorthy, S. *Nature Struct. Biol*. **7**, 693
33. Anthrax toxin lethal factor (LF) (2001). Pannifer, A.D. *et al. Nature*, **414**, 229
34. ADP-ribosylating C3 exoenzyme from *Clostridium botulinum* (2001) Han, S. *et al. J. Mol. Biol*. **305**, 95
35. *Clostridium perfringens* epsilon toxin (2004). Cole, A.R. *et al. Nature Struct. Biol*. **11**, 797
36. *Streptococcus intermedius* intermedilysin (2004). Polekhina. G. *et al. Acta Crystallogr. D Biol. Crystallogr*. **60**, 347
37. *Yersinia pseudotuberculosis* YPMa superantigen (2004). Donadini, R. *et al. Structure* **12**, 145
38. Botulinum neurotoxin type E (2004). Agarwal *et al. Biochemistry* **43**, 6637
39. *Haemophilus ducreyi* genotoxin (2004). Nesic, D. *et al. Nature* **429**, 429
40. *S. aureus* leucotoxin S component (2004). Guillet *et al. J. Biol. Chem*. **279**, 41028

**TABLE 1.3** Clostridium species

| **MAJOR PATHOGENIC SPECIES** | **Toxins** |
|---|---|
| *C. septicum* (Pasteur and Joubert, 1877) | 4 |
| *C. chauvoei* (Kitt, 1887) | 4 |
| *C. tetani* (Kitasato, 1889) | 2 |
| *C. perfringens* (Welch and Nuttall, 1892) | 17 |
| *C. novyi /oedematiens* (Novy, 1894) | 8 |
| *C. botulinum* (van Ermengem, 1895) | 3 |
| *C. histolyticum* (Weinberg and Séguin, 1916) | 5 |
| *C. bifermentans* (Weinberg and Séguin, 1919) | 3 |
| *C. sordellii* (Sordelli, 1922) | 4 |
| *C. haemolyticum* (Hall, 1929) | 3 |
| *C. difficile* (Hall and O'Toole, 1935) | 3 |
| *C. spiroforme* (Fitzgerald *et al*., 1965) | 2 |
| *C. butyricum* (Howard *et al*., 1977) | 1 |
| *C. baratii* (Hall *et al*., 1985) | 2 |
| *C. argentinense* (Suen *et al*., 1988) | 1 |
| Total | 62 |

**Five species are involved in toxin-induced gas gangrenes:**
*C. perfringens, C. septicum, C. histolyticum, C. novyi, C. sordellii*

**Four species produce neurotoxins (zinc–dependent proteinases):**
*C. botulinum, C. tetani, C. baratii, C. argentinense*

**Nine species produce StreptolysinO-related cholesterol-dependent cytolysins:**
*C. septicum, C. chauvoei, C. tetani, C. botulinum, C. perfringens, C. novyi /oedematiens, C. histolyticum, C. bifermentans, C. sordellii*

**References**: Bernheimer, A. W. (1944) Alouf and Jolivet-Reynaud (1981) Hatheway (1990); Rood and Cole (1991); Yoshihara *et al*. (1994); Songer (1996); Popoff and Marvaud (1999); Herreros *et al*. (1999); Petit *et al*. (1999); Alouf (2000); Stevens (2000); Rossetto *et al*. (2001); and Chapters 36 and 57 of this volume.

### *Clostridial gas gangrene*

*C. perfringens* and *C. septicum* are the most representative among the five pathogenic species of the clostridia that cause gas gangrene. The latter causes a form of gangrene in the absence of any external trauma, while the former is the causative agent of traumatic gangrene after deep penetration, invasion, and destruction of healthy muscles (van Heyningen, 1955; Mac Lennan, 1962; and Chapter 56 of this volume).

*Clostridium perfringens*

This microorganism is historically the main source of one of the major bacterial protein toxins, namely the lethal and hemolytic α-toxin. *C. perfringens* has been known for decades as *Clostridium welchii* in honor of the American pathologist William Henry Welch (1850–1934), who provided the first extensive description of the microorganism. Although this organism was first cultured probably as early as 1891 by Achalme, this author did not make a connnection to gangrene. One year later, Welch and Nuttal (1892) reported the discovery of what they called a strain of *Bacillus aerogenes capsulatus* found in gas-containing blood vessels during post-mortem examination of a corpse of a 38-year-old bricklayer (see Derewenda and Martin, 1998). Gangrene made its most widespread appearance 22 years later during World War I (MacPherson *et al*., 1922). Using strains from the battlefront, Bull and Pritchett (1917) conducted a series of studies that led them to conclude that "the cause of death in the infection is not a blood invasion of the microorganisms but an intoxication with definite and very potent poisons produced in the growth of the bacilli in the tissues of the body." At the end of the 1960s, the bacterium was named *C. perfringens*.

It took two decades of research to conclude that the culture broth contained a varying number of toxins, among them α-toxin, the prominent toxin of this species. As many as 17 exotoxins have been described so far in the literature (McDonel, 1980; Hatheway, 1990; Songer, 1996). A crude *C. perfringens* toxoid vaccine was demonstrated to provide protection as early as 1937 by Penfold and Tolhurst.

*Discovery and characterization of C. perfringens α-toxin*

Historically, this toxin was one of the major bacterial protein toxins. The toxin was first characterized as a lethal and hemolytic toxin by Nagler (1939), Seiffert (1939), and van Heyningen (1941), who observed its interaction with serum lipids and egg yolk suspensions. An important breakthrough was the seminal discovery by Marjorie Giffen Macfarlane and Knight (1941) that the α-toxin is "a lecithinase which decomposes lecithin into phosphocholine and a diglyceride." This finding constitutes an important landmark in toxinology and for the first time, the mode of action of a protein toxin was elucidated at the molecular level. Other discoveries by Macfarlane were soon to follow (1948, 1950), as she identified the phospholipase activity of *C. oedematiens, C. sordellii*, and *C. haemolyticum. C. perfringens* α-toxin is currently considered as the prototype of the 15 cytolytic bacterial protein toxins so far identified that disrupt eucaryotic cell membranes by hydrolysis of their constitutive phospholipids (Table 1.1). This toxin belongs to the family of the zinc metallo-phospholipases (Titball, 2000; and Chapter 27 of this volume). Its crystal structure has been recently established (Naylor *et al*., 1998).

## Group A streptococci (*Streptococcus pyogenes*)

This species produces two types of toxins:

1. The membrane-damaging cytolysins, streptolysins O and S were discovered as early as 1902 by Marmorek and clearly differentiated in the 1920s and 1930s (Neill and Mallory, 1926; Todd 1938). These toxins are described in a comprehensive review (Alouf, 1980) and in Chapters 26, 38, and 42 of this volume.
2. A series of imunocytotropic toxins of the family of superantigens (see Alouf, 1980 section IV; and Chapters 51 and 53 of this volume), the prototype of which is the streptococcal erythrogenic toxin A, which plays an important role in streptococcal pathogenicity and diseases (Cunningham *et al*., 2000; Bisno *et al*., 2003).

### *Streptococcal erythrogenic toxin*

The pioneering work of George F. Dick and Gladys Henry Dick (1924), who provided the proof that streptococci, identified later as group A streptococci (*S. pyogenes)*, are the causative agent of scarlet fever, constitutes an important hallmark in bacterial toxinology and the microbiology of infectious diseases. The Dicks were also the discoverers of the so-called "scarlet fever toxin" (Dick and Dick, 1924 a, b) recently named streptococcal pyrogenic exotoxin (SPE) by certain authors (Schlievert and Gray, 1989; Mc Shan, 1997). Three immunologically different toxin serotypes A, B, and C have been reported (Watson, 1960). Toxin B was later found to be a cysteine proteinase devoid of superantigenic properties (Gerlach *et al.,* 1994). The three toxin serotypes are produced exclusively by group A streptococci, separately, simultaneously, or in various combinations, depending on culture media and strains (Knöll *et al*., 1991).

### *Streptococcal superantigens*

Erythrogenic toxins A and C are the prototypes of the family of structurally related streptococcal superantigens discovered in the past 10 years named streptococcal superantigen (SSA), streptococcal pyrogenic exotoxins G, H, I, J, L, M, and the "streptococcal mitogenic exotoxins" (SMEZ) 2, 3, . . . 24 (Proft *et al*., 2001; Alouf and Müller-Alouf, 2003; Petersson *et al*., 2004; and Chapters 51 and 53 of this volume).

An important discovery relevant to the erythrogenic toxin was the report of Frobisher and Brown as early as 1927 that a filterable agent from scarlet fever strains of group A streptococci induced toxin production by non-scarlatinal strains. This was confirmed by Bingel (1949) and Zabriskie (1964), who found that bacteriophage from streptococcal strain T12gl converted a non-lysogenic, toxin A–negative strain to a lysogenic, toxin A–positive one. Subsequently, Nida and Ferretti (1982) showed that toxic conversion is produced by a number of temperate streptococcal bacteriophages and conversion can be effected in many streptococci. The *speA* gene encoding the erythrogenic toxin A was cloned from bacteriophage T12 (Johnson and Schlievert, 1984) and its nucleotide sequence determined by Weeks and Ferretti (1986) and Johnson *et al*. (1986), respectively.

The demonstration that erythogenic toxin was encoded by a bacteriophagic gene was an important breakthrough in the genetics of bacterial toxins, as was also the case of the discovery of the bacteriophagic origin of the gene encoding diphtheria toxin (Freeman, 1951) below as described.

## The conundrum of *Bacillus anthracis* toxin complex

This complex plays a dominant role in the pathogenesis of anthrax disease. It constitutes a particularly interesting and somewhat unusual molecular structure characterized by binary combinations of three secreted proteins: the protective antigen (PA), the lethal factor (LT), a zinc-dependent protease that cleaves six of the seven known mitogen-activated protein kinase kinases (Klimpel *et al*., 1994; Vitale *et al*., 2000), and the edema factor (EF), a calmodulin-dependent adenylate cyclase (Leppla, 1982, 1988). The association of PA and LF constitutes the lethal toxin, while that of PA and EF constitutes the edema toxin. The three proteins are encoded by *pagA*, *lef*, and *cya* genes. These genes are borne by a large (182-kbp) plasmid called pXO1 (Mock and Fouet, 2001; Koehler, 2002).

### *Historical background*

Anthrax was recognized for many centuries as a severe disease of animals and humans. It is believed to have been one of the seven plagues suffered by the Egyptians in the time of Moses and was clearly described in ancient Greece (Lincoln and Fish, 1970).

The disease occurs in two main forms: localized cutaneous infections and septicemic (Lincoln *et al*., 1964, 1970). The most characteristic superficial feature of the cutaneous infections is the black eschar that gives its name to the disease and to the causative microorganism (in Greek, anthrakos ανθρακοσ, *coal*). The study of anthrax and its causative agent *Bacillus anthracis* has attracted the attention of microbiologists since the mid 1850s. In 1850, the French physicians François Rayer (1796–1867) and Casimir Joseph Davaine (1811–1882) detected microscopic rods in the blood of animals with anthrax. These "bodies" were named "bacteridies" by Davaine (1863) who contended that these organisms were the causative agents of the disease. Koch (1876) obtained pure cultures of *B.anthracis* for the first time. Louis Pasteur (1822–1895) isolated the bacterium in broth medium from which he inoculated experimental animals who died from anthrax (Pasteur and Joubert, 1878). He also used *B. anthracis* to develop an effective attenuated live vaccine (Pasteur 1880). Since that time, the production of a toxin was postulated by many authors throughout the first half of the twentieth century, particularly in the 1930s, but no lethal toxin had been demonstrated in either anthrax bacilli or filtrates from laboratory cultures (see Smith, 2002). As early as 1907, Eisenberg reported that the supernatant of a bacterial culture injected into guinea pigs and rabbits quickly killed the animals and evoked this effect as that of a "toxin." A major breakthrough occurred as of 1953 when Harry Smith, J. Kreppie, and their coworkers started intensive research in Porton Down (United Kingdom) and successfully resolved the conundrum of anthrax toxin (Stephen, 1986). The complex steps used by the authors to isolate the toxin from the plasma or exudates from dying mice or guinea pigs previously infected with *B. anthracis* has been reviewed recently by Smith (2002). The three components of the anthrax toxin complex were soon characterized by the British group (Smith *et al*., 1956; Stanley and Smith, 1961), who called them factors I, II, and III, corresponding to the edema factor, protective antigen (PA), and lethal factor (EF) of the American authors (Lincoln and Fish, 1970). Beall *et al*. (1962) independently discovered factor III.

The production and purification of the three components of anthrax toxin has been described in detail by Leppla (1988). Since then, great progress in the purification, genetics, cell biology, biochemistry, and immunological aspects of the toxin has been achieved (see Mock and Fouet, 2001; Turnbull, 2002; Koehler, 2002; Chaudry *et al*., 2002; Mourez *et al*., 2002, Mourez,

2004; and Chapter 8 of this volume). The crystal structures of PA (Petosa *et al*., 1997) and LF (Pannifer *et al*., 2001) have been determined (see Table 1.2).

### *B. anthracis* and bioterrorism

Unfortunately, *B. anthracis* and its spores and toxins recently became agents of bioterrorism after the deliberate release of anthrax in the United States of America in September and October 2001 (Jernigan *et al*., 2001). This criminal use of this toxin completely changed the international perception of the risk of bioterrorism.

## MAJOR ACHIEVEMENTS IN TOXIN RESEARCH FROM 1975 TO DATE

The major facets of toxin research of the past 29 years concerned mainly the discovery and characterization of roughly 120 novel toxins among them:

(a) *Helicobacter pylori* vacuolating cytotoxin (see Chapter 24 of this volume ).
(b) Several *Pseudomonas aeruginosa* exotoxins: three ADP-ribosyl transferases exotoxin A, Exo S, Exo T, the adenyl cyclase Exo Y, the phospholipase Exo U (Iglewski *et al*., 1977, 1978; Yahr *et al*., 1998; Krall *et al*., 2000; Maresso *et al*., 2004; and Chapter 14 of this volume).
(c) *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) identified in 1981 by two independent groups from *S. aureus* strains isolated from patients with toxic shock syndrome (TSS) and found to be the cause of this disease (Schlievert *et al*., 1981; Bergdoll *et al*., 1981, McCormick *et al*., 2001; and Chapter 50 of this volume).
(d) Novel *S.aureus* enterotoxins and *S.pyogenes* superantingens.
(e) *Bacteroides fragilis* zinc protease enterotoxin (Van Tassel *et al*., 1992; Sears, 2001; and Chapter 28 of this volume).
(f) The cytolethal distending toxins (Johnson and Lior, 1987a, b; Pickett and Whitehouse, 1999; Thelestam and Frisan, 2004; and see Chapter 23 of this volume).
(g) The binary actin –ADP-ribosylating toxins from various species (see Chapter 12 of this volume).
(h) Novel *C. botulinum, C. baratii, C. butyricum,* and *C. argentinense* neurotoxins (Popoff and Marvaud, 1999; and Chapter 19 of this volume).
(i) *Yersinia pseudotuberculosis* superantigens YPMS for "*Y. pseudotuberculosis*-derived mitogens" (Uchiyama *et al*., 1993; Abe *et al*., 1993), designated YPMa after the discovery of two variants YPMb (Ramamurthy *et al*., 1997), and YPMc (Carnoy et *al*., 2002), respectively. YPMa was shown to behave as a virulence factor (Carnoy *et al*., 2000).
(j) *S. aureus* and *Staphylococcus hyicus* exfoliative toxins (Table 1 and Chapter 56 of this volume).
(k) *Clostridium difficile* toxins A and B (Wren, 1992; Sears and Kaper, 1996).
(l) *E. coli* shiga-like toxins (verotoxins) (see Chapter 17 of this volume).
(m) Enteric toxins from: *Aeromonas species*, *Bacillus cereus, Campylobacter jejuni, E. coli* enteroaggregative, enterohemorragic, enteroinvasive, enteropathogenic, and enterotoxigenic enterotoxins (heat-labile and heat-stable and other toxins), *Plesiomonas shigelloides, Samonella enterica* toxins, *Vibrio cholerae* and other *Vibrio* species toxins (see Sears and Kaper, 1996; Laohachai *et al*., 2003; and relevant chapters of this volume).
(n) Novel ADP-ribosyl transferases.
(o) Novel membrane-damaging toxins (Alouf, 2003).

### The cloning and determination of the nucleotide sequence of more than 200 toxin structural genes and a number of regulatory genes

The structural genes are mostly located on bacterial chromosomes (80%), but also on plasmids, bacteriophages, transposons, or pathogenicity islands (PAIs) recently considered as a subtype of genomic islands (GEIs). The discovery of the latter was a considerable breakthrough in the field of bacterial pathogenesis, since these complex genomic segments may carry genes encoding not only protein toxins but also other virulence and pathogenicity factors such as adhesins, secretion, or iron uptake systems (see Hacker and Kaper, 2000; Hacker *et al*., 2004; Chapters 2 and 3 and other relevant chapters of this volume).

Bacteriophage-borne genes encode, among other toxins, diphtheria and cholera toxin, *S. pyogenes* pyrogenic exotoxins A and C, *S. aureus* exfoliative toxin B and enterotoxins A and E, *Clostridium botulinum* neurotoxins C1 and D, and *E. coli* Shiga-like toxins I and II. Plasmid-borne genes encode tetanus neurotoxin and botulinum toxin G, anthrax toxin three-component factors (protective antigen, edema factor, lethal factor), *Enterococcus feacalis* cytolysin, *Shigella dysenteriae* enterotoxin 2, the *E. coli* RTX type enterohemorrhagic (EhxA) toxin, and both heat-labile and heat-stable enterotoxins. The latter

was also shown to be encoded by a transposon gene. On the other hand, the genetic determinants of *E. coli* alpha-hemolysin are generally found on large plasmids in animal isolates of this microorganism or on the chromosome of the strains causing urinary tract infections in humans. The gene encoding *C. perfringens* enterotoxin is located on a transposon integrated between two chromosomal house-keeping genes in human food-poisoning strains, while in animal strains the enterotoxin gene is carried on large plasmids (see relevant chapters of this volume).

***Genetic regulation of the expression of bacterial toxins***

This field witnessed recently important progress (Stibitz, 2003; Cotter and Jones, 2003; Raffestin *et al.*, 2004; and Chapter 4 of this volume and other relevant chapters). A variety of toxins and enzymes were found to be under the control of two-component regulatory systems consisting of membrane-associated sensor kinases and cytoplasmic response regulators, which are often DNA-binding proteins. This is the case for the BvgA/BvgS system for *Bordetella pertussis*, VirR/VirS for *C. perfringens*, and CovR/CovS for *S. pyogenes*. In the case of *S. aureus*, the production of various toxins and virulence factors is coordinately controlled by at least three genomic regulators, *agr* (accessory gene regulator), *sar* (staphylococcal accessory regulator), and *sae* (*S. aureus* exoprotein expression). Another interesting regulatory system concerns diphtheria toxin (DT), the synthesis of which has been known since the initial investigations of Pappenheimer and Johnson (1936) to take place in low iron concentration. As mentioned before, the structural gene for the toxin is found in a bacteriophage adjacent to the phage *att* site, mapping at the junction between the phage and bacterial chromosomal DNA. The investigations of the groups of Murphy and Holmes showed that DT synthesis is inhibited by a *trans*-acting factor encoded on *C. diphtheriae* chromosome. This led to the discovery of the bacterial *dtxR* gene encoding an 226 amino acid iron responsive repressor protein DtxR. This protein binds to the *tox* operator under conditions of high iron concentration and inhibits transcription of the *tox* gene. As mentioned above the synthesis of many other bacterial toxins and virulence factors is also negatively regulated by iron.

## Molecular structure and topology of protein toxins

The determination of the nucleotide sequence of encoding structural genes of bacterial toxins allowed the determination of the amino acid sequences of the encoded toxins, thus leading to the extraordinary development of toxin biochemistry. Most toxins are single-chain polypeptides with molecular sizes ranging from about 2–3 kDa for *E. coli* thermostable enterotoxins up to 300 kDa for *Clostridium difficile* toxins A and B, which are the largest single-chain bacterial protein toxins hitherto identified (Wren, 1992; von Eichel-Streiber *et al.*, 1996).

However, many toxins occur as oligomeric multimolecular complexes comprising two or more noncovalently bonded distinct subunits. Cholera toxin and *E. coli* heat-labile enterotoxins I and II form heterohexamers (A1-B5 complex) composed of one 28 kDa A-subunit (the ADP-ribosylating moiety of the toxin) and five identical 11.8-kDa B-subunits that allow the specific binding of the toxin to ganglioside GM1 at the surface of intestinal target cells. Shiga and *E. coli* Shiga-like toxins also form heterohexamers (A1-B5 complex) composed of a single 32-kDa A-subunit (the N-glycosidase moiety that cleaves a specific adenine residue in the 28 S rRNA component of the eucaryotic ribosomal complex) and five identical 7.7 kDa B-subunits that bind to the terminal galactose residues on globotriasoyl ceramide at the surface of target cells. Pertussis toxin produced by *Bordetella* species, the most complex structure known so far among bacterial toxins, is a multimolecular A1-B5 hexamer composed of five dissimilar subunits. Other A-B type toxins are the bipartite toxins (see Saelinger, 2003; Sandvig, 2003; and Popoff *et al.*, 1989).

## Three-dimensional crystal structure

In 1986, Allured and Collier reported the first determination of the three-dimensional (3D) structure of a crystallized bacterial toxin (*P. aeruginosa* exotoxin A). Since then, the 3D structure of more than 40 toxins has been established so far, namely that of diphtheria toxin, pertussis toxin *E. coli*, and *V. cholerae* enterotoxins, Shiga toxin, and various staphylococcal and streptococcal superantigenic toxins, anthrax toxin components, botulinum neurotoxins A, B, and E, etc. (Table 1.2). These important achievements paved the way to fathom in-depth structure-activity relationships.

## General paradigms of the mechanisms of bacterial protein toxins' action on eucaryotic cells

At the turn of the 1970s, two revolutionary paradigms heralded the onset of a new era in our understanding of the mechanism of the action of toxins at the subcellular and molecular levels on target human and animal cells. The two paradigms classified the mode of action

of the toxins into two general operational mechanisms: toxins acting ultimately on intracellular targets after crossing the cell membrane and toxins strictly acting at the surface of the cell membrane, respectively.

### *Protein toxins modifiying intracellular target molecules (the conundrum of the molecular action of A-B type toxins)*

Working with diphtheria toxin, R. J. Collier (1975, 1977) was the first to demonstrate that this toxin could enter inside cell cytosol and inactivate an intracellular target molecule leading to the impairment of cell functions. This discovery led to the paradigm that a great number of bacterial (and certain plant) protein toxins consist of two functionally different moieties designated A and B with the A part causing the intracellular damage and the B part serving to bind A to appropriate cell receptors and deliver it to the interior (Popoff, 1998 and Sandvig, 2003). This process involves endocytosis by several complex mechanisms and translocation and trafficking into the cytosol from different intracellular organelles. The A and B moieties can be located on two distinct domains of a single-chain polypeptide (e.g., diphtheria toxin, *P. aeruginosa* exotoxin A) or on two different proteins, example oligomeric toxins (e.g., cholera, pertussis, shiga, and Shiga-like toxins) or binary (bipartite) toxins consisting of two entirely distinct soluble proteins that only associate on the surface of their target cells (see Leppla: in Saelinger, 2003). The A moieties possess various enzymatic activities depending on the nature of the relevant toxins, namely ADP-ribosytransferases, adenylcyclases, metalloproteases, RNA *N*-glycosidases, glycosyl-transferases, and deamidases (see Table 1.1). Recent studies identified many toxin receptors, toxin-induced modulation of various, effectors of intracellular signal transduction pathways, and apoptotic events. The establishment in the past 15 years of the 3D crystal structure of many A-B type toxins (Table 1.2) provided a framework to understand how toxins recognize receptors, penetrate membranes, and interact with and modify intracellular substrates.

### *Protein toxins acting on cytoplasmic membrane components without intracellular penetration*

This process concerns two distinct types of toxins with totally different functional and molecular modes of action.

*Receptor-targeted toxins*

These toxins bind to appropriate cell receptors with subsequent triggering of intracellular processes via transmembrane signaling followed by harmful effects. Two main classes of toxins exhibit these properties:

1. Superantigenic toxins (see Chapters 49–53).
2. *E. coli* heat-stable ST enterotoxins and related toxins from other Gram-negative bacterial species.

These toxins bind to the guanylate cyclase receptor of target intestinal cells, leading to the activation of cellular guanylate cyclases, which are brush border membrane glycoproteins. The activation provokes an elevation of cyclic GMP that stimulates chloride secretion and/or inhibition of NaCl absorption resulting in net intestinal fluid secretion (diarrhea). A similar effect is observed with the hormone guanylin, which shares 50% homology with the toxin and activates the same receptor.

*E. coli* ST was first characterized by Kunkel and Robertson (1979) in enterotoxigenic *E. coli* (ETEC). Based on structure-function relationship, STs are divided into two distinct types, namely STa (or STI) and STb (or STII). STa differs from STb by its resistance to proteases, solubility in methanol, and its activity in the suckling mouse model. In enteroaggregative *E. coli* (EAEC), a heat-stable toxin named EAST1 was identified and shown to cause diarrhea in humans. This is often compared to STa. Apart from *E. coli,* other enteric bacteria produce ST toxins. These include *V. cholerae* O1, non O1 or non–O 139, *V. mimicus*, *Yersinia enterocolitica, Y. kristensii, Citrobacter freundii,* and *Klebsiella pneumoniae* (see Sears and Kaper, 1986; Laohachai *et al*., 2003; and Chapters 25 and 48 of this volume).

*Membrane-damaging (cytolytic) toxins*

Toxin-induced cell damage was historically identified by virtue of the cytolytic action of a great number of toxins on human and animal erythrocytes and to a lesser extent on other cells. These toxins known as *membrane-damaging toxins* (MDTs) or cytolysins produced by both Gram-positive and Gram-negative bacteria possess the ability to damage or disrupt the integrity of the plasma membrane of target cells, reflected by cell swelling and subsequently cell lysis. Many members of the MDTs are important virulence and pathogenicity factors and are sometimes lethal to humans and animals. This family of toxins, initially investigated by the pioneering studies of Alan Bernheimer over three decades since the 1940s (Bernheimer, 1944; Bernheimer and Rudy, 1986) and then by many other groups, constitutes the most important family of bacterial toxins. To date, this family comprises 117 members (35% of the whole repertoire of 337 toxins so far identified). Three types of MDTs can be distinguished, based on their action on cell membranes: (i) enzymically active cytotolysins (phospholipases), which degrade cell-membrane phospholipid bilayer (Table 1.1 and Chapter 27), (ii) tensioactive cytolysins, which solubilize certain cell-membrane components by

a detergent-like action, (iii) pore-forming cytolytic toxins (PFTs), which create channels (pores) through the cytoplasmic membrane.of targetcells. These three types of MDTs encompass 26, 10, and 81 cytolytic proteins, respectively (see Alouf, 2003). The "pore" concept (paradigm) for the last group of MDTs proposed by the end of the 1970s on biochemical and electron microscopic studies initiated by Bernheimer, Freer, Alouf, Bhakdi, and many other authors (see Alouf, 2001; van der Goot, 2003) witnessed a considerable development particularly through the determination of the 3D structures of many cytolysins (Table 1.2) .

The process of pore formation was consistent with the following *modus operandi:* the toxins are released by the bacteria as monomeric water-soluble proteins and bind to cell surface components. Toxin binding facilitates the concentration of the monomers and their transition to form non-covalently associated oligomers, leading to an amphipathic state of the oligomers followed by insertion into the membrane and the formation of protein-lined pores of various sizes, depending on the toxin involved. The various families of pore-forming toxins include particularly the RTX cytolysins ( Welch, 2001; Oxhamre and Richter-Dahlfors, 2003), *Aeromonas hydrophila* aerolysin and related toxins (Fivaz *et al*., 2001, and Cole *et al*., 2000) *S. aureus* leucocidins, alpha- and gamma-toxins (Prevost *et al*., 2001), the cholesterol-dependent cytolysins (Alouf, 2001; Billington *et al*., 2001; Tweten *et al*., 2001), the bi-component A/B-type cytolysins from *Serratia, Proteus, Edwardsiella, and Haemophilus* species (Braun and Hertle, 1999), *V. cholerae* and other *V*. species (Shinoda, 1999) (see Chapters 26 and 29 to 47 of this volume).

## RATIONAL/CANONICAL CLASSIFICATION OF BACTERIAL TOXINS

The considerable progress of our knowledge of the molecular, structural, and genetic aspects of bacterial protein toxins revealed common functional and/or molecular similarities, which allow a rational/canonical classification of these toxins into families. We briefly describe some major families.

### The family of the cytolethal distending toxins (CDTs)

This family (17 members at least) constitutes the most recently discovered group of toxins produced by a variety of Gram-negative bacteria (Thelestam and Fizan, 2001; Dreyfus *et al*., 2003; Nesic *et al*., 2004; and Chapter 23 of this volume). CDTs possess the unique ability to induce DNA double-strand breaks (DSBs) in both proliferating and nonproliferating cells, thereby causing irreversible cell cycle arrest or death of the target cells. CDTs are encoded by three linked genes (*cdtA, cdtB,* and *cdtC*). All three of these gene products are required to constitute the fully active holotoxin, and this is in agreement with the recently determined crystal structure of CDT. The toxin is endocytosed via clathrin-coated pits and requires an intact Golgi complex to exert the cytotoxic activity. Several issues remain to be elucidated regarding CDT biology, such as the detailed function(s) of the cdtA and cdtC subunits, the identity of the cell surface receptor(s) for CDT, the final steps in the cellular internalization pathway, and a molecular understanding of how CDT interacts with DNA. Moreover, the role of CDTs in the pathogenesis of diseases still remains unclear.

### The family of RNA *N*-glycosidases, glycosyltransferases, and deamidases: Shiga and Shiga-like toxins, ricin, and other plant toxins

Shiga toxin is an A-B type toxin produced by *Shigella dysenteriae* discovered in 1898 by Kiyoshi Shiga (1870–1957). The toxin is constituted by a ring of identical B-chains non-covalently associated with the A chain. The latter is responsible for the RNA *N*-glycosidase activity, which provokes the cleavage of a purine residue from eucaryotic cell 28S ribosomal RNA, resulting in blockage of protein synthesis and ultimate death of target cell (Endo *et al*., 1988; O'Loughlin and Robins-Browne, 2001; Melton-Celsa and O'Brien, 2003; and Chapter 17 of this volume). Similar structurally and functionally related toxins called *Shiga-like toxins* or *verotoxins* are produced by enterohemorragic *E. coli* (six variants: Stx 1, 1c, 2, 2c, 2d, 2e) and other bacteria such as *Aeromonas hydrophila, A. cavia, Citrobacter freundii,* and *Enterobacter cloacae*. Moreover, very similar toxins from various plants (ricin, abrin, etc.) have been widely investigated (Barbieri *et al*., 1993; Melton-Celsa and O'Brien, 2003).

### The family of the bacterial ADP-ribosylating toxins (ADP ribosyl transferases: ADPRTs)

These toxins constitute a class of functionally conserved enzymes produced by a variety of pathogenic Gram-positive and Gram-negative bacterial species, which display toxic and often lethal activity in humans and certain animals. These proteins share the ability to transfer the ADP ribose moiety of β-NAD+ to an eucaryotic target protein. This process impairs essential functions of target cells, thus modifying signal transduction pathways or causing rapid cell death (see

Pallen *et al*., 2001; Barbieri and Burns, 2003; Aktories and Barth, 2004; Masignani *et al*., 2004 for recent references; and Chapter 12 of this volume).

Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A inhibit protein synthesis by inactivating elongation factor 2 and subsequently cause cell death (Collier 1975, 1977). Other toxins in this family are *P. aeruginosa* cytotoxic exotoxin S, which uncouples Ras signal transduction, pertussis toxin, cholera toxin, and heat-labile enterotoxins LT-I and LT-II from *E. coli,* which interferes with the human host by ADP-ribosylating regulatory heterotrimreric G proteins (Barbieri and Burns, 2003 Masignani *et al*., 2004). The family of binary (bipartite) toxins include the mosquiticidal toxin (MTX) from *B. sphaericus* (Carpusca *et al*., 2004), *Clostridium botulinum* C2 toxin (Ohishi *et al*., 1980 Aktories and Barth, 2004), *C. perfringens* iota toxin (Stiles and Wilkins, 1986) Marvaud *et al*., 2001), *Clostridium spiroforme* toxin (Popoff *et al*., 1989), *C. difficile* ADP-ribosyl transferase (Perelle *et al*., 1997). Other members of the ADPRT family include the low molecular-weight (25–30 kDa) C3 and C3-like enzymes produced by *C. botulinum, C. limosum, B. cereus, S. aureus* respectively, the epidermal differentiation inhibitor (EDIN) from *S. aureus,* and the recently discovered ADPR, SpyA from *Streptococcus pyogenes*, which shares amino acid identity with EDIN and *C. botulinum* C3 (Pallen *et al*., 2001; Coye and Collins, 2004). Recently, the application of *in silico* analyses allowed the identification of more than 20 novel putative members of the ADPRT family and represents a novel challenge in the genomic era (Pallen *et al*., 2001; Masignani *et al*., 2004).

### *Common molecular features of the ADPRT family*

Bacterial ADPRTs have only limited overall amino acid sequence identity. However, the 3D crystal structure and computer modeling studies of those toxins investigated so far indicate that the active site domains of these proteins are related and contain a conserved NAD-binding catalytic domain formed of two perpendicular β-sheet cores and flanked by either one or two α-helices. Moreover, a catalytic glutamate residue is found in the active site of these molecules (Han and Tainer, 2002; Masignani *et al*., 2004).

## The family of the glycosylating and deamidating toxins

The family of the glycosylating toxins includes large single-chain proteins *C. difficile* toxins A and B, the lethal and the hemorrhagic toxins from *Clostridium sordellii*, the α-toxin from *C. novyi,* and various toxin isoforms mainly produced by *C. difficile*. These toxins share common features: they have high molecular weights (>250 kDa) and possess glucosyl or N-acetyl glucosaminyltransferase properties. They also share the same functional topology. and are about 40 to 90% identical in their amino acid residues (Aktories, 2003). The toxins inactivate Rho GTPases by glucolysation of a functionally essential threonine (Thr 37) residue. With the exception of *C. novyi* α-toxin, which uses UDP-GlcNAc, the other cytotoxins use UDP-glucose as a substrate. The toxins split the activated nucleotide sugar and transfer only one sugar moeity onto the hydroxyl group of Thr 37 leading to the mono-*O*-glucosylation or mono-*O*-*N*-acetylglucosaminylation of the eucaryotic target protein (von Eichel-Streiber *et al*., 1996; Selzer *et al*., 1996; Popoff *et al*., 1996).

Another process is the deamidation and transglutamination of Rho GTPases by the cytotoxic necrotizing factors (CNFs) 1 and 2 produced by certain *E. coli* strains and the dermonecrotizing toxin (DNT) from *Bordetella pertussis* (Flateau *et al*., 1997; Schmidt *et al*., 1997).

## The family of the superantigenic toxins

These toxins exhibit like other bacterial or viral superantigens (SAgs) highly potent, lymphocyte-transforming (mitogenic) activity toward human and/or other mammalian T lymphocytes. Unlike conventional antigens, SAgs bind to certain regions of major histocompatibility complex (MHC) class II molecules of antigen-presenting cells (APCs) outside the classical antigen-binding groove and concomitantly bind under their native form to T cells at the level of certain specific motifs of the variable region of the β chain (Vβ) of T cell receptor (TcR). This interaction triggers the activation (proliferation) of the targeted T lymphocytes and leads to the *in vivo* or *in vitro* release of high amounts of various cytokines and other effectors by immune cells. Each SAg interacts specifically with a characteristic set of Vβ motifs.The repertoire of the superantigenic toxins include: (i) the classical *S. aureus* enterotoxins A, B, C (and antigenic variants) D, E, and the recently discovered enterotoxins G to U, and toxic shock syndrome toxin-1,(ii) the *S. pyogenes* SAgs comprising the classical pyrogenic (erythrogenic) exotoxins A and C and the newly identified pyrogenic toxins G, H, I, J, L, M, as well as SMEZ and SSA, and (iii) the *Yersinia pseudotuberculosis* superantigens.

The structural and genomic aspects of these toxins and their molecular relatedness have been widely investigated (Proft *et al*., 2001; Alouf and Müller-Alouf, 2003; Petersson *et al*., 2004, and Chapters 51 to 55 of this

volume). The 3D crystal structure of many of them have been determined (Table 1.2) as well as that of certain of their complexes with MHC class II molecules and (or) TcR receptors. The production of SAgs by staphylococci and group A streptococci during host infection by these bacteria contributes to a broad spectrum of diseases ranging from mild to severe cutaneous and other tissue infections, to life-threatening septicemia, and toxic shock syndromes (McCormick *et al*., 2001; Llwelyn and Cohen, 2002; Alouf and Müller-Alouf, 2003; Krakauer, 2003; Petersson *et al*., 2004; and Chapters 49 to 53 of this volume).

## CONCLUSION

The discovery of the first and major bacterial protein toxins at the end of the nineteenth century paved the way for an outstanding development of a new field not only in microbiology but in many aspects of biological and medical sciences.

The wealth of information obtained during the past 114 years of research in bacterial toxinology has provided a great deal of knowledge regarding the molecular structure, the genetic aspects, and the interaction of these fascinating molecules with the various cellular systems and tissues of the human and animal organisms. The study of the biological effects of bacterial toxins at the subcellular and molecular levels revealed their highly complex implications in a variety of physiological and metabolic processes. The key role of bacterial toxins in the pathogenesis of various acute, chronic, and certain autoimmune diseases afforded new insights in our understanding of infectious diseases. Many questions remain unanswered as yet. Novel toxins will be discovered and new achievements will certainly emerge in the coming years, particularly the design and use of novel therapeutic strategies (drugs, vaccines, adjuvants) in the management of toxin-induced diseases and prophylaxis of susceptible populations. Moreover, several lines of experimental and clinical evidence recently allowed the design of engineered toxins for their potential use against certain tumours and virus-infected cells.

## REFERENCES


Abe, J., Takeda, T., Watanabe, H., Nakao, H., Kobayashi, N., Leung, D.Y.M., and Kohsaka, T. (1993). Evidence for superantigen production by *Yersinia pseudotuberculosis. J. Immunol*. **151,** 4183–4188.

Achalme, P. (1891). Examen bactériologique d'un cas de rhumatisme articulaire aigu mort de rhumatisme cérébral *C. R. Soc. Biol.* **3,** 651–658.

Ahrens, P. and Andresen, L.O. (2004). Cloning and sequence analysis of genes encoding *Staphylococcus hyicus* exfoliative toxin types A, B, C, and D. *J. Bacteriol*. **186,** 1833–1837.

Aktories, K. (2003). Glucolysating and deamidating bacterial protein toxins. In: *Bacterial Proteins Toxins*. (eds. D. Burns, J.T. Barbieri, B.H Iglewski, and R. Rappuoli), ASM Press, Washington, D.C., pp. 229–243.

Aktories, K. and Barth, H. (2004). Clostridium botulinum C2 toxin—New insights into the cellular up-take of the actin—ADP ribosylating toxin. *Int. J. Med. Microbiol*. **293,** 557–564.

Alouf, J.E. (1980). Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther*. **11,** 211–270.

Alouf, J. E. (2000). Pore-forming bacterial protein toxins. *Curr.Topics Microbiol. Immunol*. **257,** 1–11.

Alouf, J. E. (2001). Cholesterol–binding cytolytic protein toxins. *Int. J. Med. Microbiol*. **290,** 351–356.

Alouf, J.E. (2003). Molecular features of the cytolytic pore-forming bacterial protein toxins. *Folia Microbiol* (Prague) **48,** 5–16.

Alouf, J.E. and Raynaud, M. (1960). Suppression du pouvoir inhibiteur du fer sur la toxinogénèse diphtérique par la levure. *Ann. Inst. Pasteur*. **99,** 708–722.

Alouf, J.E. and Jolivet-Reynaud, C. (1981). Purification and characterization of *Clostridium perfringens* delta-toxin. *Infect. Immun*. **31,** 536–546.

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological, and clinical aspects. *Int. J. Med. Microbiol*. **292,** 429–440.

Arbuthnott, J.P. (1970). Staphylococcal α–toxin. In: *Microbial Toxins*. (eds. S.E. Ajl, S. Kadis, and T.C. Montie), Academic Press, New York, vol I, 189–236.

Barbieri, J.T. and Burns, D.T. (2003). Bacterial toxins that covalently modify eukaryotic proteins by ADP-ribosylation. In: *Bacterial Proteins Toxins* (eds. D. Burns, J.T. Barbieri, B.H Iglewski, and R. Rappuoli), ASM Press, Washington, D.C. pp. 215–228.

Barbieri, L., Battelli, M.G. and Stirpe, F. (1993). Ribosomes inactivating proteins from plants. *Biochim. Biophys. Acta* **1154,** 237–282.

Barksdale, L. and Arden, S.B. (1974). Persisting bacteriophage infections, lysogeny and phage conversion. *Ann. Rev. Microbiol*. **28,** 265–299.

Barth, H., Aktories, K., Popoff, M.R., and Stiles, B.G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol. Mol. Biol. Rev*. **68,** 373–402.

Beall, F.A., Taylor, M.J., and Thorne, C.B. (1962). Rapid lethal effects in rats of a third component upon fractionating the toxin of *Bacillus anthracis. J. Bacteriol*. **83,** 1274–1280.

Behring, E. and Kitasato, S. (1890). Ueber das Zustandekommen der Diphtherie–Immunität und der Tetanus–Immunität bei Thieren. *Deutsch. Med. Wochenschr*. **16,** 1113–1114.

Behring, E. (1882). Die Blutserumtherapie bei Diphtherie und Tetanus. *Z. Hyg. Infectionskrankh*. **12,** 1–9.

Bergdoll, M.S., Crass, B.A., Reiser, R.F., Robbins, R.N., and Davis, J.P. (1981). A new staphylococcal enterotoxin, enterotoxin F associated with toxic-shock syndrome *Staphylococcus aureus* isolates. *Lancet* ***i***, 1017–1021.

Bernheimer, A.W. (1944). Parallelism in the lethal and hemolytic activity of the toxin of *Cl. septicum. J. Exp. Med*. **80,** 309–320.

Bernheimer, A.W. and Rudy, B. (1986). Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta* **864,** 123–141.

Billington, S.J., Jost, B.H., and Songer, J.G. (2000). Thiol-activated cytolysins: structure, function, and role in pathogenesis. *FEMS Microbiol; Lett*. **182,** 197–205.

Bingel, K.F. (1949). Neue Untersuchungen zur Scharlachätiologie. *Deutsch. Med. Wochenschr*. **127,** 703–706.

Bisno, A.L., Brito, M.O. and Collins, C.M. (2003). Molecular basis of group A streptococcal virulence. *The Lancet* **3,** 191–199.

Braun,V. and Hertle, R. (1999). The family of *Serratia* and *Proteus* cytolysins. In: *The Comprehensive Sourcebook of Bacterial Protein*

*Toxins* (eds. J.E. Alouf and J. H Freer), Academic Press, London pp. 349–361.

Bruschettini, A. (1892). Sulla diffusione del veleno del tetano nell'organismo. *Riforma Medica* **8,** 256–259.

Bull, C.G. and Pritchett, I.W. (1917). Toxin and antitoxin of and protective inoculation against *Bacillus welchii. J. Exp. Med*. **26,** 119.

Carle, A., and Rattone, G. (1884). Studio sull'oziologia del tetano. *Giorn. Accad. Med. Torino*. **32,** 174–179.

Carnoy, C., Müller-Alouf, H., Desreumaux, P., Mullet, C., Grangette, C., and Simonet, M. (2000). The superantigenic toxin of *Yersinia pseudotuberculosis*: a novel virulence factor? *Int. J. Med. Microbiol*. **290,** 477–482.

Carnoy, C., Floquet, S., Marceau, M., Sebbane, F., Haentjens-Herwegh, S., Devalckenaere, A., and Simonet, M. (2002). The superantigen gene ypm is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis. J Bacteriol*. **184,** 4489–4499.

Carpusca, I., Schirmer, J. and Aktories, K. (2004). Two-site autoinhibition of the ADP-ribosylating mosquitocidal toxin (MTX) from *Bacillus sphaericus* by its 70-kDa ricin-like binding domain. *Biochemistry* **43,** 12009–12019.

Chaudry, G.J., Moayeri, M., Liu, S. and Leppla, S.H. (2001). Quickening the pace of anthrax research: three advances point towards possible therapies. *Trends Microbiol*. **10**, 58–62.

Cole, A.R., Gibert, M., Popoff, M., Moss, D.S, Titball, R.W. and Basak, A.K. (2000). *Clostridium perfringens* epsilon-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nature Struct. Mol. Biol*. **1,** 797–798.

Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. *Bacteriol. Rev*. **39,** 54–85.

Collier, R.J. (1977). Inhibition of protein synthesis by exotoxins from *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa*. In: *The Specificity and Action of Animal, Bacterial, and Plant Toxins* (ed. P. Cuatrecasas), Chapman and Hall, London pp. 67–98.

Confer, D.L. and Eaton, J.W. (1982). Phagocyte impotence caused by an invasive adenylate cyclase. *Science*, **217,** 948–950.

Cotter, P.A. and Jones, A.M. (2003). Phosphorylated control of virulence gene expression in *Bordetella. Trends Microbiol*. **11,** 367–373.

Coye, L.H. and Collins, C.M. (2004). Identification of SpyA, a novel ADP-ribosyltransferase of *Streptococcus pyogenes. Mol. Microbiol*. **54,** 89–98.

Cunningham, M.W. (2000). Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev*. **13,** 470–511.

Davaine, C. (1862). Recherche sur les infusoires du sang dans la maladie connue sous le nom de sang de rate. *C. R. Acad. Sci*. **57,** 220–223.

De, S.N. (1959). Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae. Nature*, **183,** 1533–1534.

De Christmas, M.J. (1888). Recherche expérimentale sur la suppuration. *Ann Inst. Pasteur*. **2,** 469–478

Derewenda, Z.S. and Martin, T.W. (1998). Structure of the α-toxin: the beauty in the beast. *Nature Structur. Biol*. **5,** 659–662.

Dick, G.F. and Dick, G.H. (1924 a). The etiology of scarlet fever. *JAMA*, **82,** 301–302.

Dick, G.F. and Dick, G.H. (1924 b). A skin test for susceptibility to scarlet fever. *JAMA*, **82,** 265–266

Dreyfus, L.A. (2003). Cytolethal distending toxins. In: *Bacterial Protein Toxins*. (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), ASM Press, Washington, D.C. pp. 257–270.

Dutta, N. K., Panse, M.W. and Kulkakarni, D.R. (1959). Role of cholera toxin in experimental cholera. *J. Bacteriol*. **78,** 594–595.

Endo, Y., Tsurgi, K., Yutsudo, T., Takeda, Y., Igasawara, T. and Igarashi, E. (1988). Site of action of a verotoxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes. *Eur. J. Biochem* **171,** 45–50.

Eisenberg, P. (1907). Sur la toxine du bacille du charbon symptomatique. *C.R. Soc. Biol*. (Paris), 613–615.

Faber, K. (1890). Die Pathogenie des Tetanos. *Berl. Klin. Wochensch*. **27,** 717–720.

Finkelstein, R.A. and LoSpalluto, J.J. (1969). Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. *J. Exp. Med*. **130,** 185–202.

Fivaz, M., Abrami, L., Tsitrin, Y. and van der Goot, F.G. (2001). *Curr. Top. Microbiol. Immunol*. **257,** 35–52 and 85–111.

Flateau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein Rho by deamidation of glutamine. *Nature*, **389,** 758–762.

Franco, A.A., Mundy, L. M., Truc, M., Wu, S., Kaperk, J.B. and Sears, S.L. (1997). Cloning and characterization of the *Bacteriodes fragilis* metalloprotease toxin gene. *Infect. Immun*. **65,** 1007–1013.

Freeman, V.J. (1951). Studies on the virulence of bacteriophage–infected strains of *Corynebacterium diphtheriae*. *J. Bacteriol*. **61,** 675–688.

Frobisher, M. Jr. and Brown, J.H. (1927). Transmissible toxinogenicity of streptococci. *Bull. Johns Hopkins Hosp*. **41,** 167–173.

Gerlach, D., Reichardt, W., Fleischer, B., and Schmidt, KH. (1994) Separation of mitogenic and pyrogenic activities from so-called erythrogenic toxin type B (Streptococcal proteinase). *Zentralbl. Bakteriol*. **280,** 507–14.

Gill, D, M., Pappenheimer, A.M.Jr., Brown, R. and Kurnick, J.J. (1969). Studies on the mode of action of diphtheria toxin. VII.Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med*. **129,** 1–21.

Glenny, A.T. and Sudmersen, H.J. (1921). Note on the production of immunity to diphtheria toxin. *J. Hyg*. (London) **20,** 176–220.

Goranson-Siekierke, J. and Holmes, R.K. (1999). Regulation of diphtheria toxin production: characterization of the role of iron and the diphtheria toxin repressor. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins*, eds. J.E. Alouf and J. H Freer. London: Academic Press, 94–103.

Groman, N.B. (1953). Evidence for the induced nature of the change from non toxinogenicity in *Corynaebacterium diphtheriae* as a result of exposure to specific bacteriophage. *J. Bacteriol*. **66,** 134–191.

Hacker, J. and Kaper, J.B. (2000). Pathogenicity islands and the evolution of microbes. *Ann. Rev. Microbiol*. **54,** 641–679.

Hacker, J., Hochhut, B., Middendorf, B., Schneider, G., Buchrieser, C., Gottschalk, G. and Dobrindt, U. (2004). Pathogenomics of mobile genetic elements of toxigenic bacteria. *Int. J. Med. Microbiol*. **293,** 453–461.

Han, S. and Tainer, J.A. (2002). The ARTT motif and a unified structural understanding of substrate recognition in ADP-ribosylating bacterial toxins and eukaryotic ADP-ribosyltransferases. *Int. J. Med. Microbiol*. **291,** 523–529.

Hanakawa, Y., Schechter, N.M., Lin, C., Nishifugi, K., Amagai, M. and Stanley, J.R. (2004). Enzymatic and molecular characteristics of the efficiency and specificity of exfoliative toxin cleavage of desmoglein 1. *J. Biol. Chem*. **279,** 5268–5277.

Hantke, K. (2001). Iron and metal regulation in bacteria. *Curr. Opin Microbiol*. **4**, 172–177.

Hatheway, C.L. (1990). Toxigenic clostridia. *Clin. Microbiol. Rev*. **3**, 66–98.

Herreros, J., Lalli, G., Montecucco, C. and Schiavo, G. (1999). Pathophysiological properties of clostridial neurotoxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J. H Freer), pp. 202–228. Academic Press, London.

Hewlett, E.L. and Gray, M.C. (2000). Adenylyl cyclase toxin from *Bordetella pertussis*. In: *Bacterial Protein Toxins* (ed. K. Aktories and I. Just), pp. 473–488. Springer Verlag, Berlin.

Hinnebusch, J., Chrepanov, P., Du, Y., Rudolph, A., Dixon, J.D., Schwan, T. and Forsberg, A. (2000). Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. *Int. J. Med. Microbiol*. **290**, 483–487.

Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyltransferase II and inhibition of protein synthesis. *J. Biol. Chem*. **243**, 3553–3555.

Iglewski, B.H., Liu, P.W. and Kabat, D. (1977). Mechanism of action of *Pseudomonas aeruginosa* exotoxin A adenosine diphosphate-ribosylation of mammalian elongation factor 2 *in vivo* and *in vitro*. *Infect. Immun*. **15**, 138–144.

Iglewski, B.H., Sadoff,J., Bjorn, M.J. and Maxwell, E.S. (1978). *Pseudomonas aeruginosa* exoenzyme S: anadenosine diphosphate-ribosyltransferase distinct from toxin A. *Proc. Natl. Acad. Sci. USA* **75**, 3211–3215.

Jepson, M. and Titball, R. (2000). Structure and function of clostridial phospholipases. *Microbes and Infection* **2**, 1277–1284.

Jernigan, J.A., Stephens, D.S., Ashford, D.A., et al. (2001). Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States. *Emerg. Infect. Dis*. **7**, 933–944.

Johnson, L.P. and Schlievert, P.M. (1984). Group A streptococcal phage T 12 carries the structural gene for pyrogenic exotoxin type A. *Mol. Gen. Genet*. **194**, 52–56.

Johnson, L.P., L'Italien, J.J. and Schlievert, P.M. (1986). Streptococcal pyrogenic exotoxin type A (scarlet fever toxin) is related to *Staphylococcus aureus* enterotoxin B. *Mol. Gen. Genet*. **203**, 354–356.

Johnson, W.M. and Lior, H. (1988a). A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. *Microb. Pathog*. **4**, 103–113.

Johnson, W.M. and Lior, H. (1988 b). A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microb. Pathog*. **4**, 115–126.

Kitasato, S. (1889). Über den Tetanusbazillus. *Z. Hyg. Infektkr*. **7**, 225–230.

Klebs, E. (1872). Beiträge zur pathologischen Anatomie der Schubwunden, Vogel, Leipzig

Klimpel, K.R., Molloy, S.S, Thomas, G.and Leppla, S.H. (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consenus sequence which is required for lethal toxin activity. *Mol. Microbiol*. **13**, 1093–1100.

Knöll, H., Shramek, J., Verbova, K., Gerlach, D., Reichardt, W. and Köhler, W. (1991). Scarlet fever and types of erythrogenic toxins produced by the infecting streptococcal strains. *Zbl. Bakt*. **276**, 94–106.

Koch, R. (1884). Ueber die Cholerabakterien. *Deut. Med. Wochenschr*. **2**, 11–13.

Koch, R. (1876). Untersuchungen über Bacterien V. Die Aetiologie der Milzbrandkrankheit, begründet auf der Entwicklungsgeschichte des Bazillus anthracis. *Beitr. z. Biol. d. Pflanzen*. **2**, 277–310.

Koehler, T.M. (2002). *Bacillus anthracis* genetics and virulence gene regulation. *Curr. Top. Microbiol. Immunol*. **271**, 143–164.

Krakauer, T. and Stiles, B.G. (2003). Staphylococcal enterotoxins, toxic shock toxin-1, and streptococcal pyrogenic exotoxins: Some basic biology of bacterial superantigens. *Recent Res. Devel. Infection & Immunity*. **1**, 1–27.

Krall, R., Schmidt, G., Aktories, K. and Barbieri, J.T. (2000). *Pseudomonas aeruginosa* ExoT is a Rho GTPase–activating protein. *Infect. Immun*. **68**, 6066–6068.

Kunkel, S.V. and Robertson, D.C. (1979). Purification and chemical characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli. Infect. Immun*. **24**, 760–769.

Ladant D. and Ullmann, A. (1999). *Bordetella pertussis* adenylate cyclase: a toxin with multiple talents. *Trends Microbiol*. **7**, 172–176.

Ladhani, S., Chapple, D.S., Joannou, C.L. and Evans, R.W. (2002). A novel method for rapid production and purification of the staphylococcal exfoliative toxins. *FEMS Lett*. **212**, 35–39.

Lalli, G., Bohnert, S., Deinhardt, K., Varestegui, C. and Schiavo, G. (2003). The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol*. **11**, 431–437.

Lamanna, C. (1990). Overview of bacterial toxins with a non reductionist approach to the mode of action of botulinal neurotoxin. In: *Microbial Toxins in Food and Feeds* (eds, A.E. Pohl. *et al*.), pp. 19–36. Plenum Press, New York.

Laohachai, K.N., Bahadi, R., Hardo, M.B., Hardo, P.G. and Kourie, J.I. (2003). The role of bacterial and non-bacterial toxins in the induction of changes in membrane transport: implications for diarrhea. *Toxicon* **42**, 687–707.

Lennox, E.S. and Kaplan A. S. (1957). Action of diphtheria toxin on cells cultivated *in vitro. Proc. Soc. Exp. Biol. Med*. **95**, 700–702.

Leppla, S.H. (1982 ). Anthrax toxin edema factor; a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. U.S.A*., **79**, 3162–3166.

Leppla, S.H. (1988). Production and purification of anthrax toxin. *Methods in enzymology* **165**, 103–116.

Leuchs, L.J. (1910). Beitraege zur Kenntnis des Toxins und Antitoxins des Bacillus botulinus. *Z. Hyg. Infektionskr*. **65**, 55–84.

Lincoln, R.E., Walker, J.S., Klein, F. and Haines, B.W. (1964). Anthrax. *Adv. Vet.Sci*. **9**, 327–368.

Lincoln, R.E. and Fish, D.C. (1970). Anthrax. In: *Microbial Toxins* (eds. T.C. Montie, S. Kadis and S.J. Ajl), pp. 361–414. Academic Press vol III, New York.

Llwelyn, M. and Cohen, J. (2002). Superantigens: microbial agents that corrupt immunity. *Lancet. Infect. Dis*. **2**, 156–162.

Locke, A. and Main, E.R. (1931). The relation of copper and iron to the production of toxin and enzyme action. *J. Infect. Dis*. **48**, 419–35.

Loeffler, F. (1884). Untersuchungen über die Bedeutung der Mikrorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe. Mitth. a. d. Kaiserl. *Gesundheitsamte*. **2**, 421–499.

Lory, S., Wolfgang, M., Lee, V. and Smith, R. (2004). The multi-talented bacterial adenylcyclases. *Int. J. Med. Microbiol*. **293**, 479–482.

Macfarlane, M.G. and Knight, B.C.J.G. (1941). The biochemistry of bacterial toxins. 1. The lecithinase activity of *Cl.welchii* toxins. *Biochem. J* . **35**, 884–902.

Macfarlane, M.G. (1948). The biochemistry of bacterial toxins: 3. The identification and immunological relations of lecithinases present in *Clostridium.oedematiens and Clostridium sordellii* toxins. *Biochem. J* . **42**, 590–595.

Macfarlane, M.G. (1950). The biochemistry of bacterial toxins. 4. The lecithinase activity of *Clostridium haemolyticum* toxin. *Biochem. J* . **47**, 250–270.

MacLennan, J.D. (1962). The histotoxic clostridial infections of man. *Bacteriol. Rev*. **26**, 177–276.

MacPherson, W.G., Bowlby, A.A., Wallace, C. and English, C. (1922). *Official History of the War*. Volume 1. Medical Services Surgery of the War. HMSO, London, p. 134.

Maresso, A.W, Baldwin, M.R. and Barbieri, J.T. (2004). Ezrin/radixin/moesin proteins are high affinity targets for ADP-ribosylation by *Pseudomonas aeruginosa Exo S. J. Biol. Chem*. **279**, 38402–38408.

Marmorek, A. (1902). La toxine streptococcique. *Ann. Inst. Pasteur* **16**, 169–178.

Marvaud, C., Smith, T., Hale, M.L., Popoff, M.R., Smith, L. and Stiles, B.G. (2001). *Clostridium perfringens* iota-toxin: mapping of receptor binding and Ia docking domains on Ib. *Infect. Immun*. **69**, 2435–2441.

Masignani, V., Balducci, E., Serruto, D., Veggi, D., Arico, B., Commanducci, M., Pizza, M. and Rappuoli, R. (2004). In silico identification of novel bacterial ADP-ribosyltransferases. *Int. J. Med. Microbiol*. **293**, 471–478.

McCormick, J.K., Yarwood, J.M. and Schlievert, P.M. (2001). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55**, 77–104.

McDonel, J.L. (1980). *Clostridium perfringens* toxins (types A, B, C, D, and E). *Pharmacol. Ther* **10**, 617–635.

McShan, W.M., Tang, Y.F. and Ferretti, J.J. (1997). Bacteriophage T12 of *Streptococcus pyogenes* integrates into the gene encoding a serine tRNA. *Mol. Microbiol*. **23**, 719–728.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, E. and de Wilde, M. (1983). Cholera toxin genes: nucleotide sequence, deletion analysis, and vaccine development. *Nature* **306**, 551–557.

Melish, M.E., Glasgow, L.A. and Turner, M.D. (1972). The staphylococcal scalded skin syndrome: isolation and partial characterization of the exfoliative toxin. *Br. J. Dermatol*. **125**, 129–140.

Melton-Celsa, A. and O'Brien, A.D. (2003). Plant and bacterial toxins as RNA-N-glycosidases. In: *Bacterial Proteins Toxins* (eds D. Burns, J.T. Barbieri, B.H Iglewski and R. Rappuoli), pp. 245–253. ASM Press, Washington, D.C.

Mock, M and Fouet, A. (2001). Anthrax. *Annu. Rev. Microbiol*. **55**, 647–671.

Mourez, M., Lacy, D.B., Cunningham, K., Legman, R., Sellman, B.R., Mogridge, J. and Collier, R.J. (2002). 2001 a year of major advances in anthrax research. *Trends Microbiol*. **10**, 287–293.

Mourez, M. (2004). Anthrax toxins. *Rev. Physiol. Biochem. Pharmacol*. **152**, 135–164.

Murphy, J.R., Michel, J.L. and Teng, M. (1978). Evidence that the regulation of diphtheria toxin production is directed at the level of transcription. *J. Bacteriol*. **135**, 511–516.

Nagler, F.P.O. (1939). Observations on a reaction between the lethal toxin of *Clostridium welchii* (type A) and human serum. *Brit. J. Exp. Pathol*. **20**, 473–485.

Naylor, C., Eton, J.T., Howells, A., Justin, N., Moss, D.S., Titball, R.W. and Basak, A.K. (1998). Structure of the key toxin in gas gangrene. *Nature Struct. Biol*. **5**, 738–746.

Neill, J.B. and Mallory, T.B. (1926). Studies on the oxidation and reduction of immunological substances. IV. Streptolysin. *J. Exp. Med*. **44**, 241–260.

Nesic, D., Hsu, Y. and Stebbins, C.E. (2004). Assembly and function of a bacterial genotoxin. *Nature* **429**, 429–433.

Nicolaier, A. (1884). Ueber infectiösen Tetanus. *Dt. med. Wochenschr*. **10**, 842–844.

Nida, S.K. and Ferretti, J.J. (1982). Phage influence on the synthesis of extracellular toxins in group A streptococci. *Infect. Immun*. **36**, 745–750.

Ohishi, I., Iwasaki, M. and Sakaguchi, G. (1980). Purification and characterization of two components of botulinum C2 toxin. *Infect. Immun*. **65**, 1402–1407.

O'Loughlin, E.V. and Robins-Browne, R.M. (2001). Effect of Shiga and Shiga-like toxins on eukaryotic cells. *Microbes Infect*. **3**, 493–507.

Oxhamre, C. and Richter-Dahlfors, A. (2003). Membrane–damaging toxins: family of RTX toxins. In: *Bacterial Protein Toxins* (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski, and R. Rappuoli), pp. 203–204. ASM Press, Washington, D.C. 203–214.

Pallen, M.J., Lam, A.C., Loman, N.J. and McBride, A. (2001). An abundance of bacterial ADP-ribosyltransferases—implications for the origin of exotoxins and their human homologues. *Trends Microbiol*. **9**, 302–307.

Pannifer, A.D., Wong, T.Y., Scawrzenbacher, R., Renatus, M., Petosa, C., Bienkowska, J. (2001). Crystal structure of the anthrax lethal factor. *Nature* **414**, 229–233.

Pappenheimer, A.M. Jr. and Johnson, S.J. (1936). Studies in diphtheria toxin production. I. The effect of iron and copper. *Br. J. Exp. Pathol*. **17**, 335–341.

Pasteur, L. and Joubert, P.A. (1877). Charbon et septicémie. Bull. *Acad. Méd*. **6**, 781.

Pasteur, L. (1880). Nouvelles observations sur l'étiologie et la prophylaxie du charbon. *C.R. Acad. Sci*. **91**, 697–701.

Payne, S.M. (2003). Regulation of bacterial toxin synthesis by iron. In: *Bacterial Toxins* (eds, D. L. Burns, J.T. Barbieri, B.H. Iglewski, and R. Rappuoli), pp. 25–38. ASM Press, Washington, D.C.

Penfold, W.J. and Tolhurst, J.C. (1937). Formol-toxoids in the prophylaxis of gas gangrene. *Med. J. Aust*. **1**, 604.

Perelle, S., Scalzo, S., Kochi, S., Mock, M. and Popoff, M. R. (1997). Immunological and functional comparison between *Clostridium perfringens* iota toxin, *C. spiroforme* toxin, and anthrax toxins. *FEMS Microbiol. Lett*. **146**, 117–121.

Petersson, K., Forsberg, G. and Walse, B. (2004). Interplay between superantigens and immunoreceptors. *Scand. J. Immunol*. **59**, 345–355.

Petit, L., Gibert, M., Popoff, M.R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol*. **7**, 104–110.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature*, **385**, 833–838.

Pickett, C.L. and Whitehouse, C.A. (1999). The cytolethal distending toxin family. *Trends Microbiol*. **7**, 292–297.

Placido-Sousa, C. and Evans, D.G. (1957). The action of diphtheria toxin on tissue cultures and its neutralization by antitoxin. *Br. J. Exp. Pathol*. **38**, 644–649.

Polekhina, G., Giddings, K.S., Tweten, R.K. and Parker, M.W. (2004). Crystallization and preliminary X-ray analysis of the human–specific toxin intermadilysin. *Acta Crystallographica D Biol. Crystallgr*. **60**, 347–349.

Pope, C.G. (1932). The production of toxin by *Corynebacterium diphtheriae*. Effect produced by addition of iron and copper to the medium. *Br. J. Exp. Pathol*. **13**, 218–223.

Popoff, M. (1998). Interactions between bacterial toxins and intestinal cells. *Toxicon*. **36**, 665–685.

Popoff, M. and Marvaud, C. (1999). Structure and genomic features of clostridial neurotoxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds J.E. Alouf and J. H Freer), pp. 174–201. Academic Press, London.

Popoff, M.R., Milward, F.W., Bancillon, B. and Boquet, P. (1989). Purification of the *Clostridium spiriforme* binary toxin and activity of the toxin on HEP2 cells. *Infect. Immun*. **57**, 2462–2469.

Prevost, G., Mourey, L., Colin, D.A. and Menestrina, G. (2001). Staphylococcal-pore-forming toxins. *Curr. Top. Microbiol. Immunol*. **257**, 53–83.

Proft, T., Arcus, V.L., Handley, V., Baker, E.N. and Fraser, J.D. (2001). Immunological and biochemical characterization of streoptococcal pyrogenic exotoxins I and J (Spe-I andSPE-J). *J. Immunol*. **166**, 6711–6719.

Raffestin, S., Marvaud, J.C., Cerrato, R., Dupuy, B. and Popoff, M.R. (2004). Organization and regulation of the neurotoxin genes in *Clostridium botulinum* and *Clostridium tetani. Anaerobe* **10**, 93–100.

Ramamurthy, T., Yoshino, K.-I., Abe, J., Ikeda, N. and Takeda, T. (1997). Purification, characterization, and cloning of a novel variant of the superantigen Yersinia *pseudotuberculosis*–derived mitogen. *FEBS Lett*. **413**, 174–176.

Ramon, G. (1923). Sur le pouvoir floculant et les propriétés immunisantes d'une toxine diphtérique rendue anatoxique (anatoxine). *C. R. Acad. Sci*. (Paris) **177**, 1338–1340.

Ramon, G. and Descombey, P.A. (1925). Sur l'immunisation antiténique et sur la production de l'antitoxine tétanique. *C.R. Soc. Biol*. (Paris). **93**, 508–598.

Rood, J.I. and Cole, S.T. (1991). Molecular genetics and pathogenesis of *Clostridium perfringens. Microb. Rev*. **55**, 621–648.

Rossetto, O., Seveso, M., Caccin P., Schiavo, G., Montecucco, C. (2001). Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon* **39**, 27–41.

Roux, E. and Yersin, A. (1888). Contribution à l'étude de la diphtérie. *Ann. Inst. Pasteur*. **2**, 629–661.

Roux, E. and Martin, L. (1894). Contribution à l'étude de la diphtérie (sérumthérapie). *Ann. Inst. Pasteur* **8**, 609–639.

Saelinger, C.B. (2004). Receptors for bacterial toxins. In: *Bacterial Protein Toxins* (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski, and Rappuoli, R), pp. 131–148. ASM Press, Washington, D.C.

Sakagushi, G. (1986). *Clostridium botulinum* toxins In: *Pharmacology of Bacterial Toxins* (eds F. Dorner and J. Drews), pp. 519–548 Pergamon Press, Oxford.

Sandvig, K. (2003). Transport of toxins across intracellular membranes. In: *Bacterial Protein Toxins* (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), pp. 157–172. ASM Press, Washington, D.C.

Schlievert, P.M., Shands, K.N., Schmid, G.P., Dan, B.B. and Nishimura, R.D. (1981). Identification and characterization of an exotoxin from *Staphylococcus aureus* assiociated with toxic-shock syndrome. *J. Infect. Dis*. **143**, 509–516.

Schlievert, P.M. and Gray, E.D. (1989). Group A streptococcal pyrogenic exotoxin (scarlet fever toxin) type and blastogen A are the same protein. *Infect. Immun*. **57**, 1865–1867.

Schmidt, G., Sehr, P.,Wilm, M., Mann, M., Just, I. and Aktories, K. (1997). Gln 63 of Rho is deamidated by *Escherichia coli* necrotizing factor-1. *Nature* **387**, 725–729.

Sears, C.L. and Kaper, J.B. (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol. Rev*. **60**, 167–215.

Sears, C.L. (2001). The toxins of *Bacteroides fragilis*. Toxicon **39**, 1737–1746.

Seiffert, G. (1939). Eine Reaktion menschlicher Sera mit Perfringenstoxin. *Z. Immun. Forsch. Exp. Ther*. **96**, 515–520.

Selzer, J., Hoffmann, F., Rex, J., Wilm, M., Mann, M. Just, I. and Aktories, K. (1996). *Clostridium novyi* α-toxin-catalyzed incorporation of GlcNac into Rho subfamily proteins. *J. Biol. Chem*. **271**, 173–177.

Shiga, K. (1898). Üeber den Dysenteriebacillus (*Bacillus dysenteriae*). Zentralbl Bakt. **24,** 817–828.

Shinoda, S. (1999). Hemolysins of *Vibrio cholerae* and other *Vibrio* species. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J. H Freer). pp. 373–385. Academic Press, London.

Smith, H. (2002). Discovery of the anthrax toxin: the beginning of studies of virulence determinants regulated *in vivo*. *Int. J. Med. Microbiol*. **291**, 411–417.

Smith, H., Tempest, D.W., Stanley, J.L., Harris-Smith, P.W. and Gallop, R.C. (1956). The chemical basis of the virulence of Bacillus anthracis. VII. Two components of the anthrax toxin: their relationship to known immunizing aggressins. *Brit. J. Exp. Pathol*. **37**, 263–271.

Songer, J.G. (1996). Clostridial enteric diseases. *Clin. Microbiol. Rev*. **9**, 216–234.

Songer, J.G. (1997). Bacterial phospholipases and their role in virulence. *Trends Microbiol*. **5**, 156–161.

Stanley, J.L. and Smith, H. (1961). Purification of fraction I and recognition of a third factor of the anthrax toxin. *J. Gen. Microbiol*. **26**, 49–66.

Stephen, J. (1986). Anthrax toxin. In: *Pharmacology of Bacterial Toxins* (eds F. Dorner and J. Drews). pp. 381–395. Pergamon Press, Oxford.

Stevens, D.L. (2000). The pathogenesis of clostridial myonecrosis. *Int. J. Med. Microbiol*. **290**, 497–502.

Stibitz, S. (2003). Two-component systems. In: *Bacterial Protein Toxins.* (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli) pp. 3–23 ASM Press, Washington D.C.

Stiles, B.G. and Wilkins, T.D. (1986). Purification and characterization of *Clostridium perfringens iota toxin*: dependence on two nonlinked proteins for biological activity. *Infect. Immun*. **54**, 683–688.

Strathdee, C.A. and Lo, R.Y. (1989). Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella hemolytica* leukotoxin determinant. *J. Bacteriol*. **171**, 916–928.

Strauss, N. and Hendee, E.D. (1959). The effect of diphtheria toxin on the metabolism of HeLa cells. *J. Exp. Med*. **109**, 144–163.

Tesh, V.L. and O'Brien, A.D. (1991). The pathogenic mechanisms of Shiga and Shiga-like toxins. *Mol. Microbiol*. **5**, 1817–1822.

Thelestam, M. and Frisan,T. (2004). Cytolethal distending toxins. *Rev. Physiol. Biochem. Pharmacol*. **152**, 111–33.

Titball, R.W., Naylor, C. E., Miller, J., Moss, D.S. and Basak, A.K. (2000). Opening of the active site of *Clostridium perfringens* α-toxin may be triggered by membrane binding. *Int. J. Med. Microbiol*. **290**, 357–361.

Tizzoni, G. and Cattani, G. (1890). Über das Tetanus Gift. Zentralbl. *Bakt*. **8**, 69–73.

Todd, E.W. (1938). The differentiation of two distinct serological varieties of streptolysin, streptolysin O, and streptolysin S. *J. Pathol. Bacteriol*. **47**, 423–445.

Turnbull, P.C. (2002). Introduction: anthrax history, disease, and ecology. *Curr. Top. Microbiol. Immunol*. **271**, 1–19.

Tweten, R.K., Parker, M.W. and Johnson, A.E. (2001). The cholesterol-dependent cytolysins (2001). *Curr. Top. Microbiol. Immunol*. **257**, 15–33.

Uchiyama, T., Miyoshi-Akiyama, T., Kato, H., Fujimaki, W., Imanishi, K. and Yan, X. J. (1993). Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* from patients manifesting acute and systemic symptoms. *J. Immunol*. **151**, 4407–4413.

van der Goot, F.G. (2003). Membrane-damaging toxins In: *Bacterial Protein Toxins* (eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), pp. 189–202. ASM Press, Washington, D.C.

van de Velde, H. (1894). Etude sur le mécanisme de la virulence du staphylocoque pyogène. *Cellule*, **10**, 401–406.

van Ermengem, E. (1897). Über einen neunen anaeroben Bacillus und seine Beziehungen zum Botulismus. *Z. Hyg. Infektkrh*. **26**, 1–56.

van Heyningen, W. E. (1941). The biochemistry of the gas gangrene toxins. I. Estimation of the a toxin of *Cl. welchii*, type A. *Biochem. J*. **35**, 1246–1256.

van Heyningen, W. E. (1950). *Bacterial Toxins.* Blackwell Scientific Publications, Oxford.

van Heyningen, W. E. (1955). The role of toxins in pathology. In: *Mechanisms of Microbial Pathogenicity*, pp. 17–34. Cambridge University Press, Cambridge.

van Heyningen, W. E. (1970). General characteristics. In: *Microbial Toxins* (eds. S.E. Ajl, S. Kadis and T.C. Montie) vol I, pp. 1–28 Academic Press, New York.

van Heynigan, W.E. and Mellanby, J. (1971). Tetanus toxin. In: *Microbial Toxins* (eds. S. Kadis, T.C. Montie and S.E. Ajl) vol IIA, pp. 69–108 Academic Press, New York.

Van Tassel, R.L., Lyerly, D.M. and Wilkins, T.D. (1992). Purification and characterization of an enterotoxin from *Bacteroides fragilis*. *Infect. Immun*. **60**, 1343–1350.

Villaseca, J.M., Navarro-Garcia, F., Mendoza-Hernandez, G., Nataro, J.M., Cravioto, A. and Eslava, C. (2000). Pet toxin from enteroaggregative *Escherichia coli* produces cellular damage associated with fodrin disruption. *Infect. Immun*. **68**, 5920–5927.

Vitale, G., Bernardi, Napolitani, G., Mock, M. and Montecucco, C. (2000). Susceptibility of mitogen-activated protein kinase kinase family members in proteolysis by anthrax lethal factor. *Biochem. J.* **352**, 739–745.

Von Eichel-Streiber, C., Boquet, P., Sauerborn, M. and Thelestam, M. (1996). Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. *Trends Microbiol*. **4**, 375–382.

von Leber, T. (1888). *Forsch. Med*. **6**, 460.

Watson, D.W. (1960). Host parasite factors in group A streptococcal infections: pyrogenic and other effects on immunologic distinct exotoxins related to scarlet fever toxins. *J. Exp. Med*. **111**, 255–283.

Weeks, C.R. and Ferretti, J.J. (1986). Nucleotide sequence of the type A pyrogenic exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T 12. *Infect. Immun*. **52**, 144–150.

Welch, W.H. and Nuttal, G.H.F. (1892). A gas producing bacillus (*Bacillus aerogenes capsulatus*, Nov. Spec.) capable of rapid development in the blood vessels after death. *Bull. Johns Hopkins Hosp*. **3**, 81–91.

Welch, R.A. (2001). RTX toxin structure and functions: a story of numerous anomalies and few analogies in toxin biology. *Curr. Top. Microbiol. Immunol*. **257**, 85–111.

Welkos, S.L. and Holmes, R.K. (1981). Regulation of toxinogenesis in *Corynebacterium diphtheriae*. I. Mutations in bacteriophage beta that alter the effects of iron on toxin production. *J. Virol*. **37**, 936–945.

White, A., Diung, X., van der Spek, J C., Murphy, J.R. and Riknge, D.R. (1998). Structure of the metal-iron-activated diphtheria toxin repressor/*tox* operator complex. *Nature* **394**, 502–506.

Wren, B.W. (1992). Molecular characterization of *Clostridium diffcile* toxins A and B. *Rev. Med. Microbiol*. **3**, 21–27.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T. *et al*. (2002). Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin ETD and EDIN-B. *Infect. Immun*. **70**, 5835–5845.

Yahr, T.L., Vallis, A.J., Hancock, M.K., Barbieri, J.T. and Frank, D.W. (1998). ExoY, an adenylate cyclase secreted by *Pseudomonas aeruginosa* type III system. *Proc. Natl. Acad. Sci. USA* **95**, 13899–13904.

Yoshihara, K., Matsushita, O., Minami, J. and Okabe, A. (1994). Cloning and nucleotide sequence analysis of the *colH* gene from *Clostridium histolyticum* encoding a collegenase and a gelatinase. *J. Bacteriol*. **176**, 6489–6496.

Zabriskie, J.B. (1964). The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. *J. Exp. Med*. **119**, 761–780.

SECTION I

# BASIC GENOMIC AND PHYSIOLOGICAL ASPECTS OF BACTERIAL PROTEIN TOXINS

CHAPTER

# 2

# Evolutionary aspects of toxin-producing bacteria

*Brenda A. Wilson and Mengfei Ho*

The toxins and effectors produced by pathogenic bacteria are located on pathogenicity islands. Increasing evidence indicates that the evolution of toxin genes involves horizontal gene transfer and recombination events mediated through plasmids, phages, transposons, and other yet-undefined transmissible elements. In addition to the host environment, toxin evolution and transmission also can occur outside the human and animal host, including in soil and aquatic environments, in the phyllosphere, and in the guts of insects, parasites, and other vectors.

## INTRODUCTION

It had long been believed that microbes capable of causing human disease evolve over long periods of time through complex interactions between the microbes and their hosts. The prevailing view was that maintenance and amplification of mutations that might lead to increased virulence required a strong or persistent selective pressure, which could only be applied specifically from exposure to the host environment. However, new evidence suggests that acquisition of large DNA segments by horizontal gene transfer (HGT) may account for a much more rapid evolution of pathogens (Groisman and Ochman, 1996), particularly in terms of the origin of virulence factors such as toxins, than was previously thought. Indeed, the abundance of toxin genes associated with "foreign" DNA segments, called *pathogenicity islands* (PAIs), in the genomes of most of the sequenced toxin-producing pathogens, compared to their non-toxigenic and/or nonpathogenic counterparts, suggests that HGT must occur at a relatively high frequency in the "real world." Consequently, scientists are beginning to ask how many of the emerging or reemerging bacterial diseases are surfacing because of the acquisition of new toxin-containing PAIs or new combinations of PAI-encoded toxin genes? And, if HGT is a major driving force in the evolution of toxins and bacterial pathogens, where is it taking place?

## MOLECULAR ECOLOGY OF TOXIN-PRODUCING BACTERIA

### Pathogenicity islands, horizontal gene transfer, and the prevalence of toxins

Strong evidence now points to an important role for HGT (in the form of mobile PAIs) in contributing to genome variability and evolution of pathogens (Miao *et al*., 1999; Boyd and Brussow, 2002; Casjens, 2003; Omelchenko *et al*., 2003; Brussow *et al*., 2004; Novichkov *et al*., 2004). The structural genes encoding most bacterial protein toxins are located on extrachromosomal plasmids or within genomes as part of putative transposons, integrated conjugative plasmids, temperate bacteriophages, or remnants of these mobile elements (see Table 2.1). Possible mechanisms by which HGT contributes to bacterial pathogenesis include exchange and recombination of toxin and other virulence genes among different bacterial populations; one-step acquisition of toxin(s) and other virulence genes to increase survival against the host's immune

**TABLE 2.1** PAI-encoded toxins proteins

| Protein Toxins | Gene | Location | Bacterial Host | Ref* |
|---|---|---|---|---|
| Diphtheria toxin (DT) | *tox* | corynephages α, β, P, π, δ, L, h, ω, (tox$^+$), γ (tox$^-$) | *Corynebacterium diphtheriae, C. ulcerans, C. pseudotuberculosis* | 1, 2, 3 |
| Clostridial neurotoxins | | | | |
| BoNT/A1 | *botA1* | Chromosome | *Clostridium botulinum* | 4, 5 |
| BoNT/A2 | *botA2* | Chromosome | *C. botulinum* | |
| BoNT/B | *botB* | Chromosome | *C. botulinum* | |
| BoNT/C1 | *botC1* | Prophage | *C. botulinum* | 6 |
| BoNT/D | *botD* | Prophage | *C. botulinum* | |
| BoNT/Dsa | *botC/D* | Phage? | *C. botulinum* | 7 |
| BoNT/E | *botE* | Plasmid, Phage | *C. botulinum, C. butyricum* | 8 |
| BoNT/F | *botF* | Chromosome | *C. botulinum, C. baratii* | 9 |
| BoNT/G | *botG* | Plasmid | *C. botulinum* | 10 |
| TeNT | *tet* | Plasmid | *C. tetani* | |
| Shiga toxin (ST) | *stx* | Phages | *Shigella dysenteriae, E. coli* | 11 |
| Shiga-like toxins (SLT) | *stx1* | ϕ 19B | *E. coli* | 12 |
| | *stx2* | ϕ933W | *E. coli* | 13 |
| | *stx2c* | ϕ2851 | *E. coli* | 14 |
| | *stx* | ϕ7888 | *S. sonnei* | 15 |
| | *stx1c* | ϕ6220 | *E. coli* | 16 |
| *Pseudomonas* cytotoxin | *ctx* | ϕCTX, PS21 | *Pseudomonas aeruginosa* | 17, 18 |
| Staphylococcal | *sea, sep* | ϕN315, ϕMu50A | *Staphylococcus aureus* | 19 |
| enterotoxins | *seg, sen, sei, sem, seo* | Enterotoxin gene cluster | | 20 |
| | *sel, sek, sec3* | TSST-1 islands | | |
| exotoxins | *set 1-15* | Exotoxin gene cluster | | |
| leukotoxins | *lukD, lukE* | Enterotoxin gene cluster | | |
| exfoliative toxins | *eta* | ϕETA | | 21 |
| | *etb* | Plasmid | | 22 |
| | *etd* | PAI | | 23 |
| leukocidins | *pvl, lukD, lukE, lukF, lukM, lukS* | ϕPVL, ϕPV83, ϕSLT | | 24, 25<br>20 |
| hemolysins | *hla, hld, hlg* | PAI | | 26 |
| toxic shock syndrome toxin-1 (TSST-1) | *tst* | TSST-1 islands | | 27 |
| Streptococcal | *ssa* | Prophages | *Streptococcus pyogenes* | 28, 29 |
| superantigen A exotoxins | *speA, speB, speC, speG, speH, speI, speK, speL, speM* | | | |
| streptolysin O (SLO) | *slo* | Chromosome | | 30 |
| streptoslysin S (SLS) | *sls* | Chromosome | | |
| Cholera toxin (CT) | *ctxAB* | CTXϕ, VPI | *Vibrio cholerae, V. mimicus* | 31, 32, 33 |
| RTX | *VcRTX* | Chromosome | *V. cholerae* | 34, 35 |
| | *VvRTX* | Chromosome | *V. vulnificus* | 36 |
| cytolethal distending toxin (CDT) | *cdtABC* | Prophage | *E. coli* | 37 |
| | | Plasmid pVir | *E. coli* | 38 |
| heat-labile enterotoxin (HLT) | *elt, etx* | Plasmid, Chromosome | *E. coli* | 39 |
| heat-stabile enterotoxins (SLT) | *estA, estB* | Transposon | | 40 |
| | *ast* (EAST-1) | Plasmid | | |
| α-hemolysin | *hly I, II* | Chromosome (PAI), Plasmid | | 41, 42 |
| enterohemolysins (Ehly 1, 2) | *eh 1, 2* | Phage | | 43, 44 |

**TABLE 2.1** Prophage-encoded toxins proteins—*cont'd*

| Protein Toxins | Gene | Location | Bacterial Host | Ref* |
|---|---|---|---|---|
| Cytotoxic necrotizing factors (CNF1, CNF2, CNFY) | *cnf1* | Chromosome (PAI) | *E. coli* | 43, 44 |
| | *cnf2* | Plasmid pVir | | 38 |
| | *cnfY* | PAI (?) | *Yersinia enterocolitica, Y. pseudotuberculosis* | 45 |
| *Pasteurella* dermonecrotic toxin (PMT) | *toxA* | λ-like linear dsDNA phage | *Pasteurella multocida* | 46 |
| Pertussis toxin (PT) | *ptx A-E* | Chromosome (?) | *Bordetella pertussis* | 47 |
| *Bordetella* Dermonecrotic toxin (DNT) | *dnt* | Chromosome (?) | *B. pertussis, B. bronchiseptica, B. parapertussis* | 48 |
| Anthrax toxins | | | *Bacillus anthracis* | |
| Protective antigen (PA) | *pag* | Plasmid (pX01) | | 49 |
| Lethal factor (LF) | *lef* | | | |
| Edema factor (EF) | *cya* | | | |

*See Table 2.2 for References.

system or to provide means for dissemination within the host or between hosts; and provision of mechanisms to enhance survival in the external environment when outside the host body.

Phages or lysogenic strains carrying toxin prophages might serve as the natural reservoir for toxin genes, with lysogenization and conversion processes taking place not only in the human or animal host such as in the lungs or intestines, but also in the external environment such as in food, water, soil, or other vectors or hosts (e.g., insects, amoeba, or plants). HGT under any of these conditions could produce new pathogenic strains and may account for the prevalence of related toxins among diverse pathogens. Examples can readily be found in some of the emerging or reemerging pathogens, including superantigen production by group A streptococci, cholera toxin production by *Vibrio cholerae*, heat-labile enterotoxin (a homologue of cholera toxin) production by enterohemorrhagic (EHEC) strains of *Escherichia coli,* and the widespread Shiga toxin-production by *Shigella* and *E. coli* strains. Indeed, studies on Shiga toxin (*stx*) gene-containing phages indicate that they are transmitted not only by temperate bacteriophages between different bacteria *in vivo*, i.e., in the intestines of humans and animals (Acheson *et al*., 1998), but also extra-intestinally in aquatic environments, such as sewage and fecally contaminated rivers and lakes (Garcia-Aljaro *et al*., 2004; Muniesa *et al*., 2004), as well as contaminated irrigation water, soil, and crops (Solomon *et al*., 2002b).

New studies indicate that phage biology may additionally contribute to bacterial pathogenesis by allowing for export or release of toxins mediated by phage lysis or for toxin gene expression upon phage induction, particularly when optimal promoters for the toxin gene are lacking in the new host. Most phage-encoded toxin genes are located near the phage attachment site, supporting acquisition by a transduction mechanism. However, the Shiga toxin genes are an exception. The genes for Stx1 and Stx2 are downstream of the λ-like transcriptional activator Q homologue and upstream of the phage lysis and morphogenesis genes in H-19B and 933W phages, respectively. Recent studies have demonstrated that expression and release of the Stx proteins from the bacteria is mediated by phage induction and lysis (Wagner *et al*., 2001; Wagner *et al*., 2002; Livny and Friedman, 2004; Tyler *et al*., 2004). This came as a surprise since several groups had previously identified a functional Fur-like promoter that was directly upstream of the *stx1* gene, and had reported that toxin expression was regulated by iron through this promoter (Calderwood *et al*., 1987; Calderwood and Mekalanos, 1987; De Grandis *et al*., 1987; Jackson *et al*., 1987). However, subsequent studies indicated that phage induction by agents such as mitomycin C dramatically increased the production and release of Stx by STEC strains far above that observed under iron regulation (Hull *et al*., 1993; Wagner *et al*., 2001; Wagner *et al*., 2002). This was further confirmed by mutational analysis of the promoter regions (Hull *et al*., 1993; Wagner *et al*., 2001; Wagner *et al*., 2002; Tyler *et al*., 2004).

Phage induction may also contribute to the release of toxins when specific protein secretion systems do not exit for the toxin or when the toxin is too large for efficient export. Phage induction is known to be triggered by bacterial stress responses, including SOS-inducing antibiotics and oxidative stress, which are known to trigger phage induction and subsequent release of Shiga toxins (Kimmitt *et al*., 2000), as well as the large clostridial toxins, ToxA and ToxB, from *Clostridium difficile* responsible for nosocomial antibiotic-induced

enterocolitis (Onderdonk *et al*., 1979; Ketley *et al*., 1984; Lyerly *et al*., 1988; Giannasca and Warny, 2004). The structural similarity of the toxin-encoding regions of different *C. difficile* strains and their absence in non-toxinogenic strains suggests that they may be part of a transmissible PAI (Braun *et al*., 1996; Hundsberger *et al*., 1997; Dupuy and Sonenshein, 1998; Mani *et al*., 2002). Another example where this may be relevant, albeit not yet demonstrated, is for the *Pasteurella multocida* toxin (PMT), whose structural gene (*toxA*) was recently shown to reside on a temperate bacteriophage (Pullinger *et al*., 2004). PMT is responsible for the symptoms of atrophic rhinitis in animals, a disease that is most prevalent and more severe when animals are under stressful conditions or during co-infections (Foged, 1992).

## Toxins encoded by plasmids, bacteriophages, and other pathogenicity islands

The clinical profile of diarrheal diseases caused by pathogenic *E. coli* strains is a composite of the various toxins and other virulence factors produced by those strains (Nataro and Kaper, 1998). Enterotoxigenic *E. coli* (ETEC) strains are characterized by the presence of two different plasmid-encoded enterotoxins, the heat-labile toxin (HLT), which is an $AB_5$-type ADP-ribosylating toxin closely related to cholera toxin, and the heat-stable toxin (HST), which is one of several related small peptide toxins produced by different strains of *E. coli* that bind and activate intestinal guanylate cyclase receptors. ETEC and extra-intestinal strains of *E. coli* from animals also often carry another Vir plasmid, which encodes the gene for cytotoxic necrotizing factor 2 (*cnf2*) (Oswald *et al*., 1991; Oswald *et al*., 1994a; Oswald *et al*., 1994b), as well as the gene for cytolethal distending toxin (*cdt*) (Peres *et al*., 1997; Toth *et al*., 2000; Mainil *et al*., 2003). Uropathogenic *E. coli* (UPEC) strains, on the other hand, carry the *cnf1* gene, which is homologous to the *cnf2* gene, but is located along with the gene for α-hemolysin (*hly*) on a chromosomal PAI (Blum *et al*., 1995). Many extra-intestinal *E. coli* strains contain variants of the α-hemolysin genes on different plasmids or chromosomal PAIs (Muller *et al*., 1983). The plasmid-encoded enterohemolysin (*ehxABD*) of enterohemorrhagic *E. coli* (EHEC) strains also has strong homology to α-hemolysin, but the N- and C-termini are different in the two proteins (Bauer and Welch, 1996; Schmidt *et al*., 1996).

Shiga toxin–producing *E. coli* (STEC) strains have emerged as a major group of food-borne pathogens (Karmali, 1989; Griffin and Tauxe, 1991; Boerlin, 1999). Nearly all of the Shiga toxin genes are carried by temperate bacteriophages (Newland and Neill, 1988; Unkmeir and Schmidt, 2000). The two classes of Shiga toxins found in STEC, Stx1 and Stx2, have similar structures and mechanisms of action. The *stx1* gene is highly conserved and nearly identical to the *stx* gene from *Shigella dysenteriae*, whereas the *stx2* gene shares ~58% DNA sequence homology with *stx1* and has many distinct variants among different STEC isolates, with some isolates containing multiple variants (Boerlin, 1999). In addition, some *stx* genes contain mosaic structures, suggesting that recombination between *stx-phages* can also occur in nature (Bastian *et al*., 1998).

The spore-forming, neurotoxin-producing *Clostridia* are strict anaerobic Gram-positive bacteria that are found ubiquitously in the environment. Neurotoxin-producing strains of *Clostridium botulinum* are defined by which one of the closely related but antigenically distinct botulinum neurotoxins (BoNT) that they produce (A1, A2, B, C1, D, E, F, or G) (Collins and East, 1998; Johnson and Bradshaw, 2001). These toxins are also related to the tetanus neurotoxin (TeNT) produced by *Clostridium tetani*. The diverse locations of the genes for these toxins are illustrative of the degree of HGT that has occurred during their evolution. Comparative phylogenic analysis has revealed the strong likelihood that HGT of BoNT genes has occurred not only within *C. botulinum* species, but also with other species, including *C. butyricum* and *C. baratti*. The genes for BoNT/A1, BoNT/A2, BoNT/B, and BoNT/F are located in gene clusters on the chromosome (Collins and East, 1998; East *et al*., 1998; Rodriguez Jovita *et al*., 1998; Santos-Buelga *et al*., 1998; Dineen *et al*., 2003); the gene for BoNT/G is plasmid encoded (Zhou *et al*., 1995); the genes for BoNT/C1 and BoNT/D are encoded by prophages (Eklund *et al*., 1971; Eklund *et al*., 1972; Hariharan and Mitchell, 1976; Hauser *et al*., 1995); and the gene for BoNT/E has been found on both phage and plasmids (Hauser *et al*., 1992; Zhou *et al*., 1993).

Some phages carrying the BoNT/C1 and BoNT/D also encode one or more C3 ADP-ribosyltransferase exoenzyme genes (Popoff *et al*., 1990; Hauser *et al*., 1993; Moriishi *et al*., 1993). Certain strains of *C. botulinum* have been reported to contain mixtures of toxin types. For example, there are reports of strains that harbor both *botB* and *botF* gene clusters, as well as strains that harbor both *botA* and *botF* gene clusters (Collins and East, 1998). Many type A strains have both the *botA1* gene cluster and a *botB* cluster, although the *botB* cluster in these cases often has a cryptic *botB* gene (Franciosa *et al*., 1994; Hutson *et al*., 1996; Rodriguez Jovita *et al*., 1998). A strain containing a chimeric gene for BoNT/Dsa, which was a composite between *botC*

and *botD* genes, has also been reported (Moriishi *et al*., 1996a; Moriishi *et al*., 1996b).

## PAI-encoded toxins delivered by type III and type IV secretion systems

A new class of PAI-encoded virulence factors includes the type III secretion systems (TTSS) and their translocated effector proteins (see Table 2.2). Although they are not called toxins per se, these effector proteins can be considered as toxins since they mediate cytotoxic effects in host cells upon being directly delivered to the host cell cytosol by the TTSS. Virulence plasmids of pathogenic *Shigella*, *E. coli*, and *Yersinia* harbor conserved clusters of genes for one or more TTSS and their associated effector proteins (Hueck, 1998; Anderson and Schneewind, 1999; Winstanley and Hart, 2001). For example, the three pathogenic strains of *Yersinia* (*Y. pestis, Y. enterocolitica*, and *Y. pseudotuberculosis*) all contain a large virulence plasmid (pYV) that encodes the TTSS and Yop (*Yersinia* outer proteins) effector proteins, which inhibit macrophage function once delivered to the macrophage by the TTSS (Cornelis *et al*., 1998). An analogous set of TTSS (Mxi/Spa) and effector (Ipa) proteins are encoded on the large invasion plasmid (pInv, pWR100 in *S. flexneri*) harbored by *Shigella* and enteroinvasive *E. coli* (EIEC) strains (Parsot and Sansonetti, 1996; Nhieu and Sansonetti, 1999; Buchrieser *et al*., 2000).

*Salmonella enterica* serovar Typhimurium harbor a number of PAIs and prophages, two of which encode TTSS and effector proteins that are directly injected into the host cell at different stages of the infection cycle (Galan and Collmer, 1999; Lostroh and Lee, 2001). The TTSS encoded by the *Salmonella* pathogenicity island-1 (SPI-1) is involved in mediating invasion of

**TABLE 2.2** Prophage-encoded effector proteins

| Effectors | Gene | Location | Bacterial Host | Ref* |
|---|---|---|---|---|
| *Pseudomonas* TTSS Effectors | | | *Pseudomonas aeruginosa* | |
| ExoS | *exoS* | Chromosome (putative PAI) | | 50 |
| ExoT | *exoT* | Chromosome (putative PAI) | | 50 |
| ExoY | *exoY* | Chromosome (putative PAI) | | 51 |
| ExoU | *exoU* | Chromosome (putative PAI) | | 52 |
| *Salmonella* TTSS Effectors | *sopE* | SopE ϕ | *Salmonella enterica* serovars | 53, 54, 55 |
| | *sopE2* | Chromosome (phage?) | Typhimurium, Typhi | |
| | *sspH2* | Chromosome (phage?) | | |
| | *gogB* | GIFSY-1 | | |
| | *ssel (gtgB, sfrH)* | GIFSY-2 | | |
| | *sspH1* | GIFSY-3 (phage remnant) | | |
| | *sipA-D, sptP, avrA* | Chromosome (SPI-1) | | |
| | *sseA-G, ssaB* | Chromosome (SPI-2) | | |
| | *sopB* | Chromosome (SPI-5) | | |
| | *sopA, slrP, sopD, sopD2, sseJ, sifA, sifB* | Chromosome | | |
| | *sopE* | Chromosome (λ-like phage remnant) | *Salmonella enterica* serovars Hadar, Gallinarum, Dublin, Enteritidis | 56 |
| *Shigella* TTSS *Effectors* | | | *Shigella flexneri* | 57 |
| Ipa proteins | *ipa A-D* | Plasmid | | |
| Enterotoxin | *senA* | Plasmid | | |
| *Yersinia* TTSS Effectors | | | | 58 |
| Yop proteins | *yopE, yopB, yopD, yopH, yopO, yopT, yopM, yopP/J* | Plasmid | *Y. pestis, Y. enterocolitica, Y. pseudotuberculosis* | |

*References for Tables 2.1 and 2.2: (1) (Buck *et al*., 1985); (2) (Ratti *et al*., 1983);(3) (Leong and Murphy, 1985); (4) (Hauser *et al*., 1995); (5) (Collins and East, 1998); (6) (Hariharan and Mitchell, 1976); (7) (Moriishi *et al*., 1996a); (8) (Zhou *et al*., 1993); (9) (East *et al*., 1998); (10) (Zhou *et al*., 1995); (11) (Unkmeir and Schmidt, 2000); (12) (Huang *et al*., 1987); (13) (Tyler *et al*., 2004); (14) (Strauch *et al*., 2004); (15) (Strauch *et al*., 2001); (16) (Koch *et al*., 2003); (17) (Hayashi *et al*., 1994); (18) (Nakayama *et al*., 1999); (19) (Novick, 2003); (20) (Kuroda *et al*., 2001); (21) (Yamaguchi *et al*., 2000); (22) (Yamaguchi *et al*., 2001); (23) (Yamaguchi *et al*., 2002); (24) (Narita *et al*., 2001); (25) (Kaneko and Kamio, 2004); (26) (Holden *et al*., 2004); (27) (Lindsay *et al*., 1998); (28) (Banks *et al*., 2002); (29) (Beres *et al*., 2002); (30) (Sierig *et al*., 2003); (31) (Waldor and Mekalanos, 1996); (32) (Boyd *et al*., 2000); (33) (Faruque *et al*., 1998); (34) (Lin *et al*., 1999); (35) (Sheahan *et al*., 2004); (36) (Chen *et al*., 2003); (37) (Janka *et al*., 2003); (38) (Oswald *et al*., 1994b); (39) (Leong *et al*., 1985); (40) (Lee *et al*., 1985); (41) (Blum *et al*., 1995); (42) (Falbo *et al*., 1992); (43) (Stroeher *et al*., 1993);(44) (Beutin *et al*., 1993); (45) (Lockman *et al*., 2002); (46) (Pullinger *et al*., 2004); (47) (Locht and Keith, 1986); (48) (Walker and Weiss, 1994); (49) (Okinaka *et al*., 1999b); (50) (Frank, 1997); (51) (Yahr *et al*., 1998); (52) (Sato and Frank, 2004); (53) (Winstanley and Hart, 2001); (54) (Zhou and Galan, 2001); (55) (Hansen-Wester and Hensel, 2001); (56) (Mirold *et al*., 2001b); (57) (Buchrieser *et al*., 2000); (58) (Cornelis *et al*., 1998)

macrophages (Wallis and Galyov, 2000), while the TTSS encoded by SPI-2 is required for survival within the macrophage and during systemic infection (Hansen-Wester and Hensel, 2001). The effector proteins injected by the SPI-1 TTSS, all of which affect various intracellular signaling processes in the macrophage, include SopB, SopE2 (encoded by a phage remnant), AvrA, SipB, SptP, SipC, SipA, SspH1 (encoded by the GIFSY-3 prophage), SspH2 (encoded by a phage remnant), SopD, SlrP, SopA, and sometimes SopE (encoded by another phage in some strains) (Galan, 2001; Zhang *et al*., 2002). The effector proteins translocated by the SPI-2 TTSS include SspH1, SspH2, SlrP, SseI, SseJ, SifA, and SifB, and they are also encoded in distinct regions on the chromosome (Miao *et al*., 1999; Miao and Miller, 2000; Galan, 2001; Zhang *et al*., 2002). Analysis of the *sopE* gene among various *S. enterica* subspecies indicates that HGT can occur between different phage families (Mirold *et al*., 2001a; Mirold *et al*., 2001b; Zhang *et al*., 2002), suggesting that shuffling of the effector protein repertoires in *Salmonella* species can and has occurred to create new epidemic strains.

## MOLECULAR EVOLUTION OF TOXINS THROUGH GENETIC EXCHANGE

### Genetic exchange and toxin evolution

If HGT is a major player in the evolution of toxin-producing pathogens, where do most of these transfers occur? Could it be that the host body is not the only setting where this type of evolution occurs? This is of particular interest because many pathogens, including those that produce toxins, spend a substantial amount of their life cycle outside the host body.

### Toxin evolution and transmission in the host

Microbes are defined by their environment, and for pathogens, that environment is believed to be predominantly the human or animal host. Pathogenic bacteria establish infections in widely diverse host environments, ranging from the skin to various mucosal surfaces such as the oral cavity, lungs, gut, and vagina. Consequently, specific host *in vivo* signals might be expected to modulate bacteria gene transfer events (Mel and Mekalanos, 1996). There are ample studies demonstrating that transfer of antibiotic resistance genes between different Gram-negative and Gram-positive bacteria can occur in the intestinal tracts of humans and animals (Nijsten *et al*., 1995; Scott and Flint, 1995; Igimi *et al*., 1996; Shoemaker *et al*., 2001). A number of genetic analyses of the plasmid profiles from clinical fecal isolates indicate that HGT most likely occurred within the human gut (Tauxe *et al*., 1989; Balis *et al*., 1996; Prodinger *et al*., 1996; Shoemaker *et al*., 2001). Conjugal gene transfer between different *Salmonella* strains has also been shown to occur within cultured human epithelial cells (Ferguson *et al*., 2002), suggesting that the phagocytic vacuoles or cytosol of animal cells could be a niche for HGT if bacteria conjugate within the intracellular environment. An interesting example of HGT can be found in the genetic evidence for homologous recombination within the vacuolating cytotoxin gene, *vacA*, which occurred between different *Helicobacter pylori* strains co-inhabiting the stomach and resulted in chimeric *vacA* genes (Atherton *et al*., 1995; Atherton *et al*., 1997; Atherton *et al*., 1999; Letley *et al*., 1999; Ji *et al*., 2002; Aviles-Jimenez *et al*., 2004).

There is considerable evidence now that continuing evolution of the Gram-positive streptococci and staphylococci is occurring via phage-mediate HGT (Hakenbeck *et al*., 2001; Kuroda *et al*., 2001; Banks *et al*., 2002; Fitzgerald *et al*., 2003; Novick, 2003). For example, Group A *Streptococcus* (GAS) strains can produce one or more of three pyrogenic exotoxins (SpeA-C), which are related to members of the so-called superantigen family found in staphylococci (Bohach *et al*., 1990). The phage-encoded genes for SpeA (the scarlet fever toxin) and SpeC are variably present in clinical isolates (Yu and Ferretti, 1989; Musser *et al*., 1991; Yu and Ferretti, 1991a; Yu and Ferretti, 1991b), and there is genetic evidence that they undergo HGT and recombination (Kapur *et al*., 1992; Beres *et al*., 2002; Smoot *et al*., 2002).

A similar evolutionary story of high variability and evidence of extensive HGT is told for the staphylococci, particularly in relation to the various toxin gene clusters for superantigens (*set*), exfoliative toxins (*eta*, *etb*), and toxic shock syndrome toxins (*tst*) found in pathogenic strains (Yamaguchi *et al*., 2000; Kuroda *et al*., 2001; Yamaguchi *et al*., 2002; Fitzgerald *et al*., 2003). The human pharyngeal cell has been shown to release a soluble factor that stimulates the lytic activation of *Streptococcus pyogenes* prophage, as well as expression of a phage-encoded extracellular DNase (Broudy *et al*., 2001; Broudy *et al*., 2002). Indeed, using a mouse model of *S. pyogenes* infection, the mammalian host was shown to promote both efficient phage induction at the mucosal surface, as well as subsequent lysogenic conversion of the non-toxigenic streptococci occupying the same niche (Broudy and Fischetti, 2003).

The vast majority of epidemic *Vibrio cholerae* strains, which are responsible for the massive diarrheal disease cholera, do not produce cholera toxin (CT) or TCP pili when they are outside of the human or animal host,

e.g., under laboratory conditions. However, all clinical isolates do make these virulence factors in the host intestines. The *ctxAB* genes that encode the A and B subunits of CT were found to be located on a putative transmissible element (called *CTX*) on the chromosome (Mekalanos *et al*., 1983; Pearson *et al*., 1993). Surprisingly, this CTX element was later demonstrated to actually be a filamentous bacteriophage (CTXϕ) that could undergo lysogenic conversion under certain laboratory conditions, but the most efficient transfer occurred in the host intestines (Waldor and Mekalanos, 1996). What was even more remarkable was the finding that CTXϕ uses as its receptor the TCP pilus, which itself is a colonization factor in the gut (Taylor *et al*., 1987; Karaolis *et al*., 1999). While classical pandemic strains of *V. cholerae* express TCP under a variety of conditions, more recent El Tor pandemic strains only express TCP in the infected mammalian host (Mekalanos *et al*., 1997). In addition, generalized transduction of CTXϕ by yet another phage (CP-T1) was found to be an alternative mechanism by which CTXϕ-carrying strains could transfer the CTX prophage into non-toxigenic strains (Boyd and Waldor, 1999).

Pathogenic *Bordetella* species (*B. pertussis, B. parapertussis,* and *B. bronchiseptica*) are closely related Gram-negative bacteria that colonize the respiratory tracts of mammals (Preston *et al*., 2004). *B. pertussis* is a strict human pathogen responsible for whooping cough; *B. parapertussis* is a recently emerged variant that infects both humans and sheep; and *B. bronchiseptica* is a broad host-range pathogen that infects primarily animals and occasionally humans. Comparative genome sequence analysis suggests that *B. pertussis* and *B. parapertussis* evolved from *B. bronchiseptica* in the recent past (Arico *et al*., 1987; Rappuoli *et al*., 1987; van der Zee *et al*., 1996a; van der Zee *et al*., 1996b; Parkhill *et al*., 2003). Interestingly, many of the genes encoding virulence factors, including the genes for pertussis toxin (*ptxA-E*), adenylate cyclase hemolysin (*cya*), dermonecrotic toxin (*dnt*), and tracheal cytotoxin (*tct*), can be found on distinct regions of the chromosomes in all three species, although some of these virulence factors, e.g., PT, may be expressed *in vitro* only by *B. pertussis* (Arico and Rappuoli, 1987). It was previously thought that PT expression differences in the strains were due to inactivating mutations in the promoter regions in *B. parapertussis* and *B. bronchiseptica* (Arico and Rappuoli, 1987). However, recent genetic analysis suggests that PT may be more tightly regulated in *B. parapertussis* and *B. bronchiseptica* and therefore is expressed only *in vivo* by these strains, while the recent mutations in the promoter region increased the transcription of the gene in *B. pertussis* (Hausman *et al*., 1996; Parkhill *et al*., 2003; Preston *et al*., 2004).

## Vaccines and toxin evolution

Despite high vaccination coverage, the once nearly eradicated diseases diphtheria and pertussis have unfortunately reemerged as a health threat in the developed countries (Bass and Stephenson, 1987; Bass and Wittler, 1994; De Serres *et al*., 1995; Galazka *et al*., 1995; Wilson, 1995; Andrews *et al*., 1997; de Melker *et al*., 1997; Baron *et al*., 1998). One possible explanation for this reemergence is that the constant selective pressure imposed by immunization might have resulted in increased antigenic divergence in the remaining bacterial population. Consequently, the effects of vaccination on toxin evolution are beginning to be examined (Pappenheimer and Murphy, 1983; Mencarelli *et al*., 1992; van der Zee *et al*., 1996b; Mooi *et al*., 1998; Guiso *et al*., 2001; Weber *et al*., 2001; von Hunolstein *et al*., 2003).

Comparative genetic analysis of the genes for *B. pertussis* PT and pertactin (an outer membrane protein) between recent epidemic isolates and the vaccine strains revealed that expansion of strains antigenically distinct from vaccine strains has occurred (Khattak and Matthews, 1993; Mooi *et al*., 1998; Weber *et al*., 2001). These findings strongly implicate vaccination as a strong driving force in the continuous evolution of the *B. pertussis* population and may forebode the emergence of novel variants resistant to vaccination. Moreover, since pertactin and PT are the primary bacterial components in acellular pertussis vaccines (ACV) that were introduced in the 1970s and have replaced the whole-cell vaccines (WCV) in some countries, these findings place into question the long-term efficacy of both ACV and WCV.

As a result of long-standing immunization programs using the diphtheria toxoid, most current isolates of *Corynebacterium diphtheriae* or *C. ulcerans* are non-toxigenic (Galazka and Robertson, 1995; Galazka, 2000a; Holmes, 2000; von Hunolstein *et al*., 2003). Of these non-toxigenic strains, most are devoid of the *tox* gene; however, these strains could potentially be converted into toxigenic strains by exposure to a corynebacteriophage β carrying the *tox* gene (Pappenheimer and Murphy, 1983; Buck *et al*., 1985; Cianciotto and Groman, 1985; Cianciotto and Groman, 1997). While there are no definite examples of this sort of genetic transfer having occurred for diphtheria, there is fear that the continued presence of non-toxigenic strains in immunized populations (Galazka and Robertson, 1995; Reacher *et al*., 2000) and the potential import of toxigenic strains from endemic areas such as Russia (Galazka, 2000b; Skogen *et al*., 2002) might allow for phage conversion to occur. In addition, it is possible that recombination events might occur within *tox*

genes between non-toxigenic strains that harbor defective *tox* genes, and, even though this has not yet been experimentally observed (Cianciotto and Groman, 1997), the possibility still remains that this could result in reversion to full toxigenicity.

## Toxin evolution and transmission in the soil environment

Many bacterial pathogens, even those for which humans or animals can serve as the natural or primary reservoir, spend a substantial amount of time outside the host body in the external environment. This suggests that the evolution of pathogens through HGT may not occur only in the host environment. For example, it is now well recognized that the selective pressure of xenobiotic pollutants in soil and water can lead to the acquisition by soil bacteria of plasmids encoding xenobiotic-degrading enzymes (Davison, 1999; Demaneche *et al*., 2001b; Ashelford *et al*., 2003). There is even some evidence that natural electro-transformation, as might occur during a thunder-lightning storm, might be a feasible mechanism for increasing the frequency of HGT in soil microcosms (Demaneche *et al*., 2001a).

*Bacillus* species are common spore-forming soil bacteria barely distinguishable at the genome sequence level (Helgason *et al*., 2000; Read *et al*., 2003; La Duc *et al*., 2004), yet this group of bacteria differs considerably in their virulence properties. *B. subtilis* is commonly used as a nonpathogenic laboratory model system for studying bacterial sporulation; *B. thuringiensis* produces a number of insecticidal toxins widely used as pesticides in agriculture and more recently in genetically modified plants to confer insect resistance; *B. cereus* is a food-borne pathogen capable of causing human and animal gastrointestinal disease; and *B. anthracis* is a human and animal pathogen that causes anthrax disease and has received much attention recently because of its potential use as a bioterror agent.

The structural genes encoding the major virulence factors of *B. anthracis* responsible for anthrax, the anthrax lethal toxin and edema toxin genes (*pag, lef, cya*) and the poly-D-glutamate capsule biosynthetic genes (*capBCA*), reside on two large plasmids, pXO1 and pXO2, respectively (Okinaka *et al*., 1999a; Okinaka *et al*., 1999b). Loss of the pXO2 plasmid resulted in the greatly attenuated Sterne vaccine strain. Although it does not appear that these plasmids are self-transmissible, there are reports that suggest conjugative plasmids from other *Bacillus* species might be able to supply the conjugal transfer functions in trans for these two virulence plasmids (Andrup *et al*., 1996; Pannucci *et al*., 2002). If this is true, then it is conceivable that other *Bacillus* species may serve as an environmental reservoir for the anthrax toxin genes. And, indeed, HGT is reported to be quite common among *Bacillus* species (La Duc *et al*., 2004), with an intriguing example being the finding that several of the *B. anthracis* pXO1 genes have homologues on the *B. cereus* chromosome Helgason *et al*., 2000) and that pXO1 contains 15 ORFs with sequence similarity to transposases, integrases, or recombinases, as well as a number of insertion (IS) elements with high homology to IS sequences found in *B. thuringiensis* and *B. cereus* (Okinaka *et al*., 1999a; Okinaka *et al*., 1999b). However, within the *B. anthracis* group there appears to be surprisingly little genetic variation, as illustrated by sequence analysis of the protective antigen toxin gene, *pag*, which revealed only five point mutations among 26 diverse *B. anthracis* isolates (Price *et al*., 1999). This finding was consistent with a previous report that used chromosomal markers (Keim *et al*., 1999), and may reflect a very recent origin of this species or other unknown population constrictions that warrant further study (Keim *et al*., 1999; Okinaka *et al*., 1999a; Okinaka *et al*., 1999b).

*B. thuringiensis* produces a considerable arsenal of toxins directed against insects and nematodes, with multiple toxin-encoding genes on plasmids and various mobilizable genetic elements on the chromosome (de Maagd *et al*., 2003). HGT, combined with recombination and shuffling between toxin genes (resulting in domain swapping) and sequence divergence, has yielded a wide range of host specificities for these insecticidal toxins (Lee *et al*., 1995; Bravo, 1997; de Maagd *et al*., 2001). The genes encoding the crystal protein toxins, for example, are frequently clustered on different transmissible plasmids or transposable elements (Schnepf *et al*., 1998; de Maagd *et al*., 2001), and conjugation between different strains has been observed in the soil environment and within insect guts (Thomas *et al*., 2001). Individual toxins have insecticidal activity only against a limited range of insect species, i.e., usually only within certain insect orders. The composite of the toxins produced by a particular strain thus defines the total insecticidal specificity and activity spectrum of that bacterial strain (de Maagd *et al*., 2001).

## Toxin evolution and transmission in aquatic environments

Other studies have shown that the concentrations of bacteriophages in natural aquatic environments can be high enough to be conducive for phage transduction (Miller, 2001; Paul *et al*., 2002), but can be especially high in contaminated water systems (Davison, 1999; Chee-Sanford *et al*., 2001; Teuber, 2001). A recent study

showed that a broad range of *E. coli* strains harboring *stx2*, and to a lesser extent *stx1*, genes are commonly found in municipal sewage and various animal wastewaters (Garcia-Aljaro *et al*., 2004). Moreover, of the *E. coli* strains in this study testing positive for toxin genes (only one out of 59 representative strains was typed as O157:H7), about 50% of those from animal wastewater and 10% of those from human sewage were also able to produce the toxin proteins. These findings not only indicate the presence of a significant exchange of these genes between bacterial populations in these environments, but also suggest a potential health risk in that the bacteria also make the toxins.

There is now strong genetic evidence that epidemic strains of *Vibrio cholerae*, the etiological agent of cholera, have been acquiring CT, TCP, and other virulence factor genes through bacteriophage transduction and conjugal transfer (Faruque *et al*., 1998; Beaber *et al*., 2002; Davis and Waldor, 2003). As mentioned above, the genes encoding CT (ctxAB) are transferred by the bacteriophage CTXϕ (Waldor and Mekalanos, 1996; Davis and Waldor, 2003). Moreover, the genes encoding the bacterial toxin co-regulated pili (TCP) that are the receptors for CTXϕ are also encoded by another putative prophage, VPIϕ (Karaolis *et al*., 1999; Davis and Waldor, 2003). Recent studies support that HGT has occurred between *V. cholerae* and other environmental *Vibrio* species, such as *V. mimicus* (Boyd *et al*., 2000), suggesting that these environmental strains might serve as reservoirs for genetic exchange, thereby contributing to the emergence of new toxigenic *Vibrio* strains.

Since HGT occurs at maximal frequencies when bacterial densities are high (Hausner and Wuertz, 1999; Hohnstock *et al*., 2000; Lilley *et al*., 2003), one might ask how genetic exchange can occur in aquatic environments, where *Vibrio* spend most of their time and where their concentrations are expected to be too low to be conducive to HGT. Studies have shown, however, that most bacteria found in marine environments are adherent to marine animals or particles, such as zooplankton or marine snow (Azam and Long, 2001). Indeed, *Vibrio* strains are often found in close association with zooplankton (Chiavelli *et al*., 2001) as dense bacterial biofilms (Watnick *et al*., 1999). This finding correlates with the observation that zooplankton blooms frequently precede cholera outbreaks and that simple water filtration to remove zooplankton can significantly reduce the incidence of cholera cases in endemic areas (Colwell *et al*., 2003). Similar findings have been observed for other environmental bacteria. The presence of particulate matter in aqueous environments was found to increase *Pseudomonas*-phage interactions and thereby stimulate transduction (Ripp *et al*., 1994; Miller, 2001).

## Toxin evolution and transmission in the phyllosphere

Other environmental settings could also serve as ideal locations for biofilm formation and consequent HGT (Davison, 1999; Miller, 2001). One such important setting is the phyllosphere, i.e., plant surfaces. Numerous examples of relatively high conjugal transfer rates on the phylloplane of plants have been observed within and between species of *Pseudomonas* and *Rhizobium*, especially when humidity was high (Davison, 1999). The soil bacterium *Ralstonia solanacearum*, a plant pathogen, even develops a state of natural competence in plant tissues and readily exchanges genetic material (Bertolla and Simonet, 1999). Aggregation or clustering of the bacteria into microhabitats in the interstices and stomata of the leaf surface has been found to stimulate bacterial survival and conjugal transfer (Normander *et al*., 1998).

Evidence for phage-mediated transduction among bacterial strains on the phylloplane has also been documented for the plant and opportunistic human pathogen *Pseudomonas aeruginosa* (Kidambi *et al*., 1994) and the indigenous plant microbes, *P. fluorescens* and *Serratia liquefaciens* (Ashelford *et al*., 2000; Ashelford *et al*., 2002; Ashelford *et al*., 2003). The transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants has been demonstrated (Solomon *et al*., 2002a; Solomon *et al*., 2002b). Indeed, the toxin-producing bacteria were found not only on the plant surfaces, but also throughout the root system and within the edible portions of the plant tissues. The implications of this, in terms of the spread of human disease through fresh produce, are alarming, and may account for a number of reported outbreaks. For instance, irrigation water contaminated with the feces of cattle from an adjacent field was implicated as the source of *E. coli* O137:H7 during a multi-state outbreak associated with consumption of mesclun lettuce (Hilborn *et al*., 1999).

## Toxin evolution in biofilms and regulation by quorum sensing

Evidence is accumulating that the high rates of HGT observed in bacterial biofilms may be attributable to quorum sensing control of conjugation and production of exoenzymes/toxins and other virulence factors, in addition to biofilm formation (Zhang *et al*., 1993; Fuqua and Winans, 1994; Hausner and Wuertz, 1999; de Kievit and Iglewski, 2000; Brelles-Marino and Bedmar, 2001). The finding that *Roseobacter* species and other bacteria associated with marine snow also produce acyl homoserine lactones (Gram *et al*., 2002) suggests

that environmental microbes may use quorum sensing to regulate phenotypic traits (e.g., biofilm formation, metabolite/toxin production, conjugation) only when their populations in the marine snow environment reach adequate densities.

The strong conservation of virulence determinants (e.g., toxins) among clinical and environmental isolates of *P. aeruginosa* supports the notion that conserved selective pressures for the maintenance of the virulence traits exist in the environmental reservoir (Wolfgang *et al*., 2003). Recent evidence suggests that quorum sensing regulation of the formation of biofilms and the production of toxins may have played a critical evolutionary advantage in the environment by providing a protective mechanism through which the bacteria are able to resist flagellate and other protozoan predation and thereby persist outside the human host (Matz *et al*., 2004). Similarly, the effector protein toxins that are delivered by the Icm/Dot type IV secretion system of *Legionella pneumophila* appear to be equally effective in mammalian cells and in amoeba (Hilbi *et al*., 2001; Molmeret *et al*., 2002).

Another well-characterized example of biofilm and toxin regulation through quorum sensing can be found for the opportunistic pathogen *P. aeruginosa* in the lungs of cystic fibrosis (CF) patients, where *P. aeruginosa* forms both aerobic and anaerobic biofilms. Each type of biofilm that *P. aeruginosa* forms differs significantly in its virulence and phenotypic properties (Hassett *et al*., 2002; Yoon *et al*., 2002; Hogardt *et al*., 2004; Matz *et al*., 2004). In the CF lung, quorum-sensing systems regulate the formation of these two types of biofilms (Singh *et al*., 2000; Yoon *et al*., 2002), as well as toxin/exoenzyme production (Hogardt *et al*., 2004). Interestingly, whereas the expression of exotoxin A (ExoA) and various protease and phospholipase exoenzymes are up-regulated by quorum sensing in biofilms, the type III secretion system and its translocated effectors (ExoS, ExoT, ExoY, and ExoU), which are only important when the bacteria are in direct contact with the host cells, are down-regulated by quorum sensing (Hogardt *et al.*, 2004).

One exception to the strong conservation of virulence genes in *P. aeruginosa* is the presence of the genes for ExoS and ExoU, with isolates from different clinical and environmental settings possessing one or the other of the *exoS* or *exoU* genes, but not both (Yahr *et al*., 1996; Feltman *et al*., 2001; Wolfgang *et al*., 2003). It has been suggested that one possible explanation may be that expression and delivery of one or the other toxin might provide a selective advantage in a particular, as yet unidentified, target host or tissue site (Wolfgang *et al*., 2003).

### Toxin evolution in the guts of insects and other vectors

New evidence is accumulating that bacteriophages and other mobile genetic elements could also play important evolutionary roles in bacterial endosymbiont systems of insect hosts (Werren *et al*., 1995; Heath *et al*., 1999; Vavre *et al*., 1999; Masui *et al*., 2000; Sandstrom *et al*., 2001; Dyson *et al*., 2002; Kondo *et al*., 2002; Hoffmeister and Martin, 2003; Fujii *et al*., 2004). There is significantly elevated frequency of HGT between bacteria while within the digestive vacuoles of protozoan cells such as *Tetrahymena pyriformis* (Schlimme *et al*., 1995). The bacteriophage WO from parasitic arthropod bacterial symbionts (*Wolbachia*) was shown to undergo recent lateral transfers between *Wolbachia* strains that co-infect a diverse set of insect host cells (Wernegreen, 2002; Wernegreen *et al*., 2003; Degnan *et al*., 2004; Fujii *et al*., 2004). Plasmid transfer from *Erwinia herbicola* to *E. cloacae* was observed in the guts of silkworms (Watanabe and Sato, 1998).

Both conjugative and mobilizable plasmids from *E. coli* could likewise be transferred to a wide variety of *Proteobacteria* in the gut of the soil microarthropod (Hoffmann *et al*., 1998), which could play host to HGT of toxin genes in *E. coli*-contaminated water or soil. *Yersinia pestis*, the causative agent of plague, has also been demonstrated to be capable of interacting with and acquiring plasmids from *E. coli* in the midgut of the flea at transfer frequencies of ~$10^{-3}$ per recipient (Hinnebusch *et al*., 2002). One might ask whether toxin gene-encoding plasmids or mobile elements might also be transferred within the guts of insects, and, indeed, such transfers can and have occurred. For example, efficient gene transfer of plasmids encoding δ-endotoxin has been observed between different strains of *Bacillus thuringiensis* in various arthropod hosts (Jarrett and Stephenson, 1990).

## CONCLUSION

The evolution of bacterial toxins involves HGT and recombination through exposure to a combination of both the host and external environments. Most toxin genes are located on PAIs on plasmids or in the bacterial chromosome as prophages or transmissible elements. Genome sequencing and genetic analysis of toxin genes in epidemiological isolates may provide more information on the extent and limitations, as well as regulatory mechanisms that influence HGT and their impact on toxin evolution.

## REFERENCES


Acheson, D.W., Reidl, J., Zhang, X., Keusch, G.T., Mekalanos, J.J. and Waldor, M.K. (1998). *In vivo* transduction with shiga toxin 1-encoding phage. *Infection & Immunity* **66**, 4496–8.

Anderson, D.M. and Schneewind, O. (1999). Type III machines of Gram-negative pathogens: injecting virulence factors into host cells and more. *Current Opinion in Microbiology* **2**, 18–24.

Andrews, R., Herceg, A. and Roberts, C. (1997). Pertussis notifications in Australia, 1991 to 1997. *Communicable Diseases Intelligence* **21**, 145–8.

Andrup, L., Jorgensen, O., Wilcks, A., Smidt, L. and Jensen, G.B. (1996). Mobilization of "nonmobilizable" plasmids by the aggregation-mediated conjugation system of *Bacillus thuringiensis*. *Plasmid*, **36**, 75–85.

Arico, B., Gross, R., Smida, J. and Rappuoli, R. (1987). Evolutionary relationships in the genus *Bordetella*. *Molecular Microbiology* **1**, 301–8.

Arico, B. and Rappuoli, R. (1987). *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. *Journal of Bacteriology* **169**, 2847–53.

Ashelford, K.E., Day, M.J. and Fry, J.C. (2003). Elevated abundance of bacteriophage infecting bacteria in soil. *Applied & Environmental Microbiology* **69**, 285–9.

Ashelford, K.E., Fry, J.C., Bailey, M.J. and Day, M.J. (2002). Characterization of *Serratia* isolates from soil, ecological implications, and transfer of *Serratia proteamaculans* subsp. quinovora *Serratia quinivorans* corrig., sp. nov. *International Journal of Systematic & Evolutionary Microbiology* **52**, 2281–9.

Ashelford, K.E., Norris, S.J., Fry, J.C., Bailey, M.J. and Day, M.J. (2000). Seasonal population dynamics and interactions of competing bacteriophages and their host in the rhizosphere. *Applied & Environmental Microbiology* **66**, 4193–9.

Atherton, J.C., Cao, P., Peek, R.M., Jr., Tummuru, M.K., Blaser, M.J. and Cover, T.L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry* **270**, 17771–7.

Atherton, J.C., Peek, R.M., Jr., Tham, K.T., Cover, T.L. and Blaser, M.J. (1997). Clinical and pathological importance of heterogeneity in *vacA*, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology* **112**, 92–9.

Atherton, J.C., Sharp, P.M., Cover, T.L., Gonzalez-Valencia, G., Peek, R.M., Jr., Thompson, S.A., Hawkey, C.J. and Blaser, M.J. (1999). Vacuolating cytotoxin (*vacA*) alleles of *Helicobacter pylori* comprise two geographically widespread types, m1 and m2, and have evolved through limited recombination. *Current Microbiology* **39**, 211–8.

Aviles-Jimenez, F., Letley, D.P., Gonzalez-Valencia, G., Salama, N., Torres, J. and Atherton, J.C. (2004). Evolution of the *Helicobacter pylori* vacuolating cytotoxin in a human stomach. *Journal of Bacteriology* **186**, 5182–5.

Azam, F. and Long, R.A. (2001). Sea snow microcosms. *Nature* **414**, 495.

Balis, E., Vatopoulos, A.C., Kanelopoulou, M., Mainas, E., Hatzoudis, G., Kontogianni, V., Malamou-Lada, H., Kitsou-Kiriakopoulou, S. and Kalapothaki, V. (1996). Indications of *in vivo* transfer of an epidemic R plasmid from *Salmonella enteritidis* to *Escherichia coli* of the normal human gut flora. *Journal of Clinical Microbiology* **34**, 977–9.

Banks, D.J., Beres, S.B. and Musser, J.M. (2002). The fundamental contribution of phages to GAS evolution, genome diversification, and strain emergence. *Trends in Microbiology* **10**, 515–21.

Baron, S., Njamkepo, E., Grimprel, E., Begue, P., Desenclos, J.C., Drucker, J. and Guiso, N. (1998). Epidemiology of pertussis in French hospitals in 1993 and 1994: thirty years after a routine use of vaccination. *Pediatric Infectious Disease Journal* **17**, 412–8.

Bass, J.W. and Stephenson, S.R. (1987). The return of pertussis. *Pediatric Infectious Disease Journal* **6**, 141–4.

Bass, J.W. and Wittler, R.R. (1994). Return of epidemic pertussis in the United States. *Pediatric Infectious Disease Journal* **13**, 343–5.

Bastian, S.N., Carle, I. and Grimont, F. (1998). Comparison of 14 PCR systems for the detection and subtyping of *stx* genes in Shiga-toxin-producing *Escherichia coli*. *Research in Microbiology* **149**, 457–72.

Bauer, M.E. and Welch, R.A. (1996). Association of RTX toxins with erythrocytes. *Infection & Immunity* **64**, 4665–72.

Beaber, J.W., Burrus, V., Hochhut, B. and Waldor, M.K. (2002). Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. *Cellular & Molecular Life Sciences* **59**, 2065–70.

Beres, S.B., Sylva, G.L., Barbian, K.D., Lei, B., Hoff, J.S., Mammarella, N.D., Liu, M.Y., Smoot, J.C., Porcella, S.F., Parkins, L.D., Campbell, D.S., Smith, T.M., McCormick, J.K., Leung, D.Y., Schlievert, P.M. and Musser, J.M. (2002). Genome sequence of a serotype M3 strain of group A *Streptococcus:* phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10078–83.

Bertolla, F. and Simonet, P. (1999). Horizontal gene transfers in the environment: natural transformation as a putative process for gene transfers between transgenic plants and microorganisms. *Research in Microbiology* **150**, 375–84.

Beutin, L., Stroeher, U.H. and Manning, P.A. (1993). Isolation of enterohemolysin (Ehly2)-associated sequences encoded on temperate phages of *Escherichia coli*. *Gene* **132**, 95–9.

Blum, G., Falbo, V., Caprioli, A. and Hacker, J. (1995). Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and alpha-hemolysin form the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. *FEMS Microbiology Letters* **126**, 189–95.

Boerlin, P. (1999). Evolution of virulence factors in Shiga-toxin-producing *Escherichia coli*. *Cellular & Molecular Life Sciences* **56**, 735–41.

Bohach, G.A., Fast, D.J., Nelson, R.D. and Schlievert, P.M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Critical Reviews in Microbiology* **17**, 251–72.

Boyd, E.F. and Brussow, H. (2002). Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. *Trends in Microbiology* **10**, 521–9.

Boyd, E.F., Moyer, K.E., Shi, L. and Waldor, M.K. (2000). Infectious CTXPhi and the vibrio pathogenicity island prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. *Infection & Immunity* **68**, 1507–13.

Boyd, E.F. and Waldor, M.K. (1999). Alternative mechanism of cholera toxin acquisition by *Vibrio cholerae*: generalized transduction of CTXPhi by bacteriophage CP-T1. *Infection & Immunity* **67**, 5898–905.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. and von Eichel-Streiber, C. (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181**, 29–38.

Bravo, A. (1997). Phylogenetic relationships of *Bacillus thuringiensis* delta-endotoxin family proteins and their functional domains. *Journal of Bacteriology* **179**, 2793–801.

Brelles-Marino, G. and Bedmar, E.J. (2001). Detection, purification, and characterization of quorum-sensing signal molecules in plant-associated bacteria. *Journal of Biotechnology* **91**, 197–209.

Broudy, T.B. and Fischetti, V.A. (2003). *In vivo* lysogenic conversion of Tox(–) *Streptococcus pyogenes* to Tox(+) with lysogenic Streptococci or free phage. *Infection & Immunity* **71**, 3782–6.

Broudy, T.B., Pancholi, V. and Fischetti, V.A. (2001). Induction of lysogenic bacteriophage and phage-associated toxin from group a streptococci during coculture with human pharyngeal cells. *Infection & Immunity* **69**, 1440–3.

Broudy, T.B., Pancholi, V. and Fischetti, V.A. (2002). The *in vitro* interaction of *Streptococcus pyogenes* with human pharyngeal cells induces a phage-encoded extracellular DNase. *Infection & Immunity* **70**, 2805–11.

Brussow, H., Canchaya, C. and Hardt, W.D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology & Molecular Biology Reviews* **68**, 560–602.

Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., D'Hauteville, H., Kunst, F., Sansonetti, P. and Parsot, C. (2000). The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Molecular Microbiology* **38**, 760–71.

Buck, G.A., Cross, R.E., Wong, T.P., Loera, J. and Groman, N. (1985). DNA relationships among some *tox*-bearing corynebacteriophages. *Infection & Immunity* **49**, 679–84.

Calderwood, S.B., Auclair, F., Donohue-Rolfe, A., Keusch, G.T. and Mekalanos, J.J. (1987). Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 4364–8.

Calderwood, S.B. and Mekalanos, J.J. (1987). Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the fur locus. *Journal of Bacteriology* **169**, 4759–64.

Casjens, S. (2003). Prophages and bacterial genomics: what have we learned so far? *Molecular Microbiology* **49**, 277–300.

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N. and Mackie, R.I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Applied & Environmental Microbiology* **67**, 1494–502.

Chen, C.Y., Wu, K.M., Chang, Y.C., Chang, C.H., Tsai, H.C., Liao, T.L., Liu, Y.M., Chen, H.J., Shen, A.B., Li, J.C., Su, T.L., Shao, C.P., Lee, C.T., Hor, L.I. and Tsai, S.F. (2003). Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Research* **13**, 2577–87.

Chiavelli, D.A., Marsh, J.W. and Taylor, R.K. (2001). The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Applied & Environmental Microbiology* **67**, 3220–5.

Cianciotto, N. and Groman, N. (1985). A beta-related corynebacteriophage which lacks a *tox* allele but can acquire it by recombination with converting phage. *Infection & Immunity* **49**, 32–5.

Cianciotto, N.P. and Groman, N.B. (1997). Characterization of bacteriophages from *tox*-containing, non-toxigenic isolates of *Corynebacterium diphtheriae*. *Microbial Pathogenesis* **22**, 343–51.

Collins, M.D. and East, A.K. (1998). Phylogeny and taxonomy of the food-borne pathogen *Clostridium botulinum* and its neurotoxins. *Journal of Applied Microbiology* **84**, 5–17.

Colwell, R.R., Huq, A., Islam, M.S., Aziz, K.M., Yunus, M., Khan, N.H., Mahmud, A., Sack, R.B., Nair, G.B., Chakraborty, J., Sack, D.A. and Russek-Cohen, E. (2003). Reduction of cholera in Bangladeshi villages by simple filtration. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1051–5.

Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M.P. and Stainier, I. (1998). The virulence plasmid of *Yersinia*, an antihost genome. *Microbiology & Molecular Biology Reviews* **62**, 1315–52.

Davis, B.M. and Waldor, M.K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. *Current Opinion in Microbiology* **6**, 35–42.

Davison, J. (1999). Genetic exchange between bacteria in the environment. *Plasmid* **42**, 73–91.

De Grandis, S., Ginsberg, J., Toone, M., Climie, S., Friesen, J. and Brunton, J. (1987). Nucleotide sequence and promoter mapping of the *Escherichia coli* Shiga-like toxin operon of bacteriophage H-19B. *Journal of Bacteriology* **169**, 4313–9.

de Kievit, T.R. and Iglewski, B.H. (2000). Bacterial quorum sensing in pathogenic relationships. *Infection & Immunity* **68**, 4839–49.

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N. and Schnepf, H.E. (2003). Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annual Review of Genetics* **37**, 409–33.

de Maagd, R.A., Bravo, A. and Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics* **17**, 193–9.

de Melker, H.E., Conyn-van Spaendonck, M.A., Rumke, H.C., van Wijngaarden, J.K., Mooi, F.R. and Schellekens, J.F. (1997). Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. *Emerging Infectious Diseases* **3**, 175–8.

De Serres, G., Boulianne, N., Douville Fradet, M. and Duval, B. (1995). Pertussis in Quebec: ongoing epidemic since the late 1980s. *Canada Communicable Disease Report* **21**, 45–8.

Degnan, P.H., Lazarus, A.B., Brock, C.D. and Wernegreen, J.J. (2004). Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of *Camponotus* species and their endosymbionts, *Candidatus blochmannia*. *Systematic Biology* **53**, 95–110.

Demaneche, S., Bertolla, F., Buret, F., Nalin, R., Sailland, A., Auriol, P., Vogel, T.M. and Simonet, P. (2001a). Laboratory-scale evidence for lightning-mediated gene transfer in soil. *Applied & Environmental Microbiology* **67**, 3440–4.

Demaneche, S., Kay, E., Gourbiere, F. and Simonet, P. (2001b). Natural transformation of *Pseudomonas fluorescens and Agrobacterium tumefaciens* in soil. *Applied & Environmental Microbiology* **67**, 2617–21.

Dineen, S.S., Bradshaw, M. and Johnson, E.A. (2003). Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. *Current Microbiology* **46**, 345–52.

Dupuy, B. and Sonenshein, A.L. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Molecular Microbiology* **27**, 107–20.

Dyson, E.A., Kamath, M.K. and Hurst, G.D. (2002). *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (*Lepidoptera: Nymphalidae*): evidence for horizontal transmission of a butterfly male killer. *Heredity* **88**, 166–71.

East, A.K., Bhandari, M., Hielm, S. and Collins, M.D. (1998). Analysis of the botulinum neurotoxin type F gene clusters in proteolytic and nonproteolytic *Clostridium botulinum* and *Clostridium barati*. *Current Microbiology* **37**, 262–8.

Eklund, M.W., Poysky, F.T. and Reed, S.M. (1972). Bacteriophage and the toxigenicity of Clostridium botulinum type D. *Nature—New Biology* **235**, 16–7.

Eklund, M.W., Poysky, F.T., Reed, S.M. and Smith, C.A. (1971). Bacteriophage and the toxigenicity of *Clostridium botulinum* type C. *Science* **172**, 480–2.

Falbo, V., Famiglietti, M. and Caprioli, A. (1992). Gene block encoding production of cytotoxic necrotizing factor 1 and hemolysin in *Escherichia coli* isolates from extraintestinal infections. *Infection & Immunity* **60**, 2182–7.

Faruque, S.M., Albert, M.J. and Mekalanos, J.J. (1998). Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiology & Molecular Biology Kevious*. **62**, 1301–14.

Feltman, H., Schulert, G., Khan, S., Jain, M., Peterson, L. and Hauser, A.R. (2001). Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. *Microbiology* **147**, 2659–69.

Ferguson, G.C., Heinemann, J.A. and Kennedy, M.A. (2002). Gene transfer between *Salmonella enterica* serovar Typhimurium inside epithelial cells. *Journal of Bacteriology* **184**, 2235–42.

Fitzgerald, J.R., Reid, S.D., Ruotsalainen, E., Tripp, T.J., Liu, M., Cole, R., Kuusela, P., Schlievert, P.M., Jarvinen, A. and Musser, J.M. (2003). Genome diversification in *Staphylococcus aureus*: Molecular evolution of a highly variable chromosomal region encoding the staphylococcal exotoxin-like family of proteins. *Infection & Immunity* **71**, 2827–38.

Foged, N.T. (1992). *Pasteurella multocida* toxin. The characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. *Acta Pathologica Microbiologica et Immunologica Scandinavia. Supplementum* **25**, 1–56.

Franciosa, G., Ferreira, J.L. and Hatheway, C.L. (1994). Detection of type A, B, and E botulism neurotoxin genes in *Clostridium botulinum* and other *Clostridium species* by PCR: evidence of unexpressed type B toxin genes in type A toxigenic organisms. *Journal of Clinical Microbiology* **32**, 1911–7.

Frank, D.W. (1997). The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Molecular Microbiology*. **26**, 621–9.

Fujii, Y., Kubo, T., Ishikawa, H. and Sasaki, T. (2004). Isolation and characterization of the bacteriophage WO from *Wolbachia,* an arthropod endosymbiont. *Biochemical & Biophysical Research Communications* **317**, 1183–8.

Fuqua, W.C. and Winans, S.C. (1994). A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *Journal of Bacteriology* **176**, 2796–806.

Galan, J.E. (2001). *Salmonella* interactions with host cells: type III secretion at work. *Annual Review of Cell & Developmental Biology* **17**, 53–86.

Galan, J.E. and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**, 1322–8.

Galazka, A. (2000a). The changing epidemiology of diphtheria in the vaccine era. *Journal of Infectious Diseases* **181** Suppl 1, S2–9.

Galazka, A. (2000b). Implications of the diphtheria epidemic in the Former Soviet Union for immunization programs. *Journal of Infectious Diseases* **181** Suppl 1, S244–8.

Galazka, A.M. and Robertson, S.E. (1995). Diphtheria: changing patterns in the developing world and the industrialized world. *European Journal of Epidemiology* **11**, 107–17.

Galazka, A.M., Robertson, S.E. and Oblapenko, G.P. (1995). Resurgence of diphtheria. *European Journal of Epidemiology* **11**, 95–105.

Garcia-Aljaro, C., Muniesa, M., Jofre, J. and Blanch, A.R. (2004). Prevalence of the stx2 gene in coliform populations from aquatic environments. *Applied & Environmental Microbiology* **70**, 3535–40.

Giannasca, P.J. and Warny, M. (2004). Active and passive immunization against *Clostridium difficile* diarrhea and colitis. *Vaccine* **22**, 848–56.

Gram, L., Grossart, H.P., Schlingloff, A. and Kiorboe, T. (2002). Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. *Applied & Environmental Microbiology* **68**, 4111–6.

Griffin, P.M. and Tauxe, R.V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli,* and the associated hemolytic uremic syndrome. *Epidemiologic Reviews* **13**, 60–98.

Groisman, E.A. and Ochman, H. (1996). Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* **87**, 791–4.

Guiso, N., Boursaux-Eude, C., Weber, C., Hausman, S.Z., Sato, H., Iwaki, M., Kamachi, K., Konda, T. and Burns, D.L. (2001). Analysis of *Bordetella pertussis* isolates collected in Japan before and after introduction of acellular pertussis vaccines. *Vaccine* **19**, 3248–52.

Hakenbeck, R., Balmelle, N., Weber, B., Gardes, C., Keck, W. and de Saizieu, A. (2001). Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of *Streptococcus pneumoniae*. *Infection & Immunity* **69** 2477–86.

Hansen-Wester, I. and Hensel, M. (2001). *Salmonella* pathogenicity islands encoding type III secretion systems. *Microbes & Infection* **3**, 549–59.

Hariharan, H. and Mitchell, W.R. (1976). Observations on bacteriophages of *Clostridium botulinum* type C isolates from different sources and the role of certain phages in toxigenicity. *Applied & Environmental Microbiology* **32**, 145–58.

Hassett, D.J., Cuppoletti, J., Trapnell, B., Lymar, S.V., Rowe, J.J., Yoon, S.S., Hilliard, G.M., Parvatiyar, K., Kamani, M.C., Wozniak, D.J., Hwang, S.H., McDermott, T.R. and Ochsner, U.A. (2002). Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Advanced Drug Delivery Reviews* **54**, 1425–43.

Hauser, D., Gibert, M., Boquet, P. and Popoff, M.R. (1992). Plasmid localization of a type E botulinal neurotoxin gene homologue in toxigenic *Clostridium butyricum* strains, and absence of this gene in non-toxigenic *C. butyricum* strains. *FEMS Microbiology Letters* **78**, 251–5.

Hauser, D., Gibert, M., Eklund, M.W., Boquet, P. and Popoff, M.R. (1993). Comparative analysis of C3 and botulinal neurotoxin genes and their environment in *Clostridium botulinum* types C and D. *Journal of Bacteriology* **175**, 7260–8.

Hauser, D., Gibert, M., Marvaud, J.C., Eklund, M.W. and Popoff, M.R. (1995). Botulinal neurotoxin C1 complex genes, clostridial neurotoxin homology, and genetic transfer in *Clostridium botulinum*. *Toxicon* **33**, 515–26.

Hausman, S.Z., Cherry, J.D., Heininger, U., Wirsing von Konig, C.H. and Burns, D.L. (1996). Analysis of proteins encoded by the *ptx* and *ptl* genes of *Bordetella bronchiseptica* and *Bordetella parapertussis*. *Infection & Immunity* **64**, 4020–6.

Hausner, M. and Wuertz, S. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative *in situ* analysis. *Applied & Environmental Microbiology* **65**, 3710–3.

Hayashi, T., Matsumoto, H., Ohnishi, M., Yokota, S., Shinomiya, T., Kageyama, M. and Terawaki, Y. (1994). Cytotoxin-converting phages, phi CTX and PS21, are R pyocin-related phages. *FEMS Microbiology Letters*. **122**, 239–44.

Heath, B.D., Butcher, R.D., Whitfield, W.G. and Hubbard, S.F. (1999). Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. *Current Biology* **9**, 313–6.

Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. and Kolsto (2000). *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Applied & Environmental Microbiology* **66**, 2627–30.

Hilbi, H., Segal, G. and Shuman, H.A. (2001). Icm/dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Molecular Microbiology* **42**, 603–17.

Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L. (1999). A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Archives of Internal Medicine* **159**, 1758–64.

Hinnebusch, B.J., Rosso, M.L., Schwan, T.G. and Carniel, E. (2002). High-frequency conjugative transfer of antibiotic resistance genes to *Yersinia pestis* in the flea midgut. *Molecular Microbiology* **46**, 349–54.

Hoffmann, A., Thimm, T., Droge, M., Moore, E.R., Munch, J.C. and Tebbe, C.C. (1998). Intergeneric transfer of conjugative and mobilizable plasmids harbored by *Escherichia coli* in the gut of the soil microarthropod *Folsomia candida* (Collembola). *Applied & Environmental Microbiology* **64**, 2652–9.

Hoffmeister, M. and Martin, W. (2003). Interspecific evolution: microbial symbiosis, endosymbiosis, and gene transfer. *Environmental Microbiology* **5**, 641–9.

Hogardt, M., Roeder, M., Schreff, A.M., Eberl, L. and Heesemann, J. (2004). Expression of *Pseudomonas aeruginosa* exoS is controlled by quorum sensing and RpoS. *Microbiology* **150**, 843–51.

Hohnstock, A.M., Stuart-Keil, K.G., Kull, E.E. and Madsen, E.L. (2000). Naphthalene and donor cell density influence field conjugation of naphthalene catabolism plasmids. *Applied & Environmental Microbiology* **66**, 3088–92.

Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S.D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K.D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B.G., Spratt, B.G. and Parkhill, J. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulen. Proceedings of the National Academy of Sciences of the United States of America **101**, 9786–91.

Holmes, R.K. (2000). Biology and molecular epidemiology of diphtheria toxin and the tox gene. *Journal of Infectious Diseases* **181** Suppl 1, S156–67.

Huang, A., Friesen, J. and Brunton, J.L. (1987). Characterization of a bacteriophage that carries the genes for production of Shiga-like toxin 1 in *Escherichia coli*. *Journal of Bacteriology* **169**, 4308–12.

Hueck, C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology & Molecular Biology Reviews* **62**, 379–433.

Hull, A.E., Acheson, D.W., Echeverria, P., Donohue-Rolfe, A. and Keusch, G.T. (1993). Mitomycin immunoblot colony assay for detection of Shiga-like toxin-producing *Escherichia coli* in fecal samples: comparison with DNA probes. *Journal of Clinical Microbiology* **31**, 1167–72.

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M. and von Eichel-Streiber, C. (1997). Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile*. *European Journal of Biochemistry* **244**, 735–42.

Hutson, R.A., Zhou, Y., Collins, M.D., Johnson, E.A., Hatheway, C.L. and Sugiyama, H. (1996). Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences. *Journal of Biological Chemistry* **271**, 10786–92.

Igimi, S., Ryu, C.H., Park, S.H., Sasaki, Y., Sasaki, T. and Kumagai, S. (1996). Transfer of conjugative plasmid pAM beta 1 from *Lactococcus lactis* to mouse intestinal bacteria. *Letters in Applied Microbiology* **23**, 31–5.

Jackson, M.P., Newland, J.W., Holmes, R.K. and O'Brien, A.D. (1987). Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. *Microbial Pathogenesis* **2**, 147–53.

Janka, A., Bielaszewska, M., Dobrindt, U., Greune, L., Schmidt, M.A. and Karch, H. (2003). Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. *Infection Immunity* **71**, 3634–8.

Jarrett, P. and Stephenson, M. (1990). Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. *Applied & Environmental Microbiology* **56**, 1608–14.

Ji, X., Frati, F., Barone, S., Pagliaccia, C., Burroni, D., Xu, G., Rappuoli, R., Reyrat, J.M. and Telford, J.L. (2002). Evolution of functional polymorphism in the gene coding for the *Helicobacter pylori* cytotoxin. *FEMS Microbiology Letters* **206**, 253–8.

Johnson, E.A. and Bradshaw, M. (2001). *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. *Toxicon* **39**, 1703–22.

Kaneko, J. and Kamio, Y. (2004). Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. *Bioscience, Biotechnology, and Biochemistry*. **68**, 981–1003.

Kapur, V., Nelson, K., Schlievert, P.M., Selander, R.K. and Musser, J.M. (1992). Molecular population genetic evidence of horizontal spread of two alleles of the pyrogenic exotoxin C gene (speC) among pathogenic clones of *Streptococcus pyogenes*. *Infection & Immunity* **60**, 3513–7.

Karaolis, D.K., Somara, S., Maneval, D.R., Jr., Johnson, J.A. and Kaper, J.B. (1999). A bacteriophage encoding a pathogenicity island, a type-IV pilus, and a phage receptor in cholera bacteria. *Nature* **399**, 375–9.

Karmali, M.A. (1989). Infection by verocytotoxin-producing *Escherichia coli*. *Clinical Microbiology Reviews* **2**, 15–38.

Keim, P., Klevytska, A.M., Price, L.B., Schupp, J.M., Zinser, G., Smith, K.L., Hugh-Jones, M.E., Okinaka, R., Hill, K.K. and Jackson, P.J. (1999). Molecular diversity in *Bacillus anthracis*. *Journal of Applied Microbiology* **87**, 215–7.

Ketley, J.M., Haslam, S.C., Mitchell, T.J., Stephen, J., Candy, D.C. and Burdon, D.W. (1984). Production and release of toxins A and B by *Clostridium difficile*. *Journal of Medical Microbiology*. **18**, 385–91.

Khattak, M.N. and Matthews, R.C. (1993). A comparison of the DNA fragment patterns of the mouse-virulent challenge strains and clinical isolates of *Bordetella pertussis*. *Journal of Infection* **27**, 119–24.

Kidambi, S.P., Ripp, S. and Miller, R.V. (1994). Evidence for phage-mediated gene transfer among *Pseudomonas aeruginosa* strains on the phylloplane. *Applied & Environmental Microbiology* **60**, 496–500.

Kimmitt, P.T., Harwood, C.R. and Barer, M.R. (2000). Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. *Emerging Infectious Diseases* **6**, 458–65.

Koch, C., Hertwig, S. and Appel, B. (2003). Nucleotide sequence of the integration site of the temperate bacteriophage 6220, which carries the Shiga toxin gene *stx(1ox3)*. *Journal of Bacteriology*. **185**, 6463–6.

Kondo, N., Nikoh, N., Ijichi, N., Shimada, M. and Fukatsu, T. (2002). Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14280–5.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–40.

La Duc, M.T., Satomi, M., Agata, N. and Venkateswaran, K. (2004). gyrB as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. *Journal of Microbiological Methods* **56**, 383–94.

Lee, C.H., Hu, S.T., Swiatek, P.J., Moseley, S.L., Allen, S.D. and So, M. (1985). Isolation of a novel transposon which carries the *Escherichia* coli enterotoxin STII gene. *Journal of Bacteriology* **162**, 615–20.

Lee, M.K., Young, B.A. and Dean, D.H. (1995). Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. *Biochemical & Biophysical Research Communications* **216**, 306–12.

Leong, D. and Murphy, J.R. (1985). Characterization of the diphtheria *tox* transcript in *Corynebacterium diphtheriae* and *Escherichia coli*. *Journal of Bacteriology* **163**, 1114–9.

Leong, J., Vinal, A.C. and Dallas, W.S. (1985). Nucleotide sequence comparison between heat-labile toxin B-subunit cistrons from Escherichia coli of human and porcine origin. *Infection & Immunity* **48**, 73–7.

Letley, D.P., Lastovica, A., Louw, J.A., Hawkey, C.J. and Atherton, J.C. (1999). Allelic diversity of the *Helicobacter pylori* vacuolating cytotoxin gene in South Africa: rarity of the vacA s1a genotype and natural occurrence of an s2/m1 allele. *Journal of Clinical Microbiology* **37**, 1203–5.

Lilley, A.K., Bailey, M.J., Barr, M., Kilshaw, K., Timms-Wilson, T.M., Day, M.J., Norris, S.J., Jones, T.H. and Godfray, H.C. (2003). Population dynamics and gene transfer in genetically modified bacteria in a model microcosm. *Molecular Ecology* **12**, 3097–107.

Lin, W., Fullner, K.J., Clayton, R., Sexton, J.A., Rogers, M.B., Calia, K.E., Calderwood, S.B., Fraser, C. and Mekalanos, J.J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proceedings of the National Agency of Sciences of the United States of America* **96**, 1071–6.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N. and Novick, R.P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology* **29,** 527–43.

Livny, J. and Friedman, D.I. (2004). Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. *Molecular Microbiology* **51,** 1691–704.

Locht, C. and Keith, J.M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232,** 1258–64.

Lockman, H.A., Gillespie, R.A., Baker, B.D. and Shakhnovich, E. (2002). *Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor. *Infection & Immunity* **70,** 2708–14.

Lostroh, C.P. and Lee, C.A. (2001). The Salmonella pathogenicity island-1 type III secretion system. *Microbes & Infection* **3,** 1281–91.

Lyerly, D.M., Krivan, H.C. and Wilkins, T.D. (1988). *Clostridium difficile:* its disease and toxins. *Clinical Microbiology Reviews* **1,** 1–18.

Mainil, J.G., Jacquemin, E. and Oswald, E. (2003). Prevalence and identity of *cdt*-related sequences in necrotoxigenic *Escherichia coli*. *Veterinary Microbiology* **94,** 159–65.

Mani, N., Lyras, D., Barroso, L., Howarth, P., Wilkins, T., Rood, J.I., Sonenshein, A.L. and Dupuy, B. (2002). Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. *Journal of Bacteriology* **184,** 5971–8.

Masui, S., Kamoda, S., Sasaki, T. and Ishikawa, H. (2000). Distribution and evolution of bacteriophage WO in *Wolbachia*, the endosymbiont causing sexual alterations in arthropods. *Journal of Molecular Evolution* **51,** 491–7.

Matz, C., Bergfeld, T., Rice, S.A. and Kjelleberg, S. (2004). Microcolonies, quorum sensing, and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. *Environmental Microbiology* **6,** 218–26.

Mekalanos, J.J., Rubin, E.J. and Waldor, M.K. (1997). Cholera: molecular basis for emergence and pathogenesis. *FEMS Immunology & Medical Microbiology* **18,** 241–8.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D., Harford, N., Groyne, F. and de Wilde, M. (1983). Cholera toxin genes: nucleotide sequence, deletion analysis, and vaccine development. *Nature* **306,** 551–7.

Mel, S.F. and Mekalanos, J.J. (1996). Modulation of horizontal gene transfer in pathogenic bacteria by *in vivo* signals. *Cell* **87,** 795–8.

Mencarelli, M., Zanchi, A., Cellesi, C., Rossolini, A., Rappuoli, R. and Rossolini, G.M. (1992). Molecular epidemiology of nasopharyngeal corynebacteria in healthy adults from an area where diphtheria vaccination has been extensively practiced. *European Journal of Epidemiology* **8,** 560–7.

Miao, E.A. and Miller, S.I. (2000). A conserved amino acid sequence directing intracellular type III secretion by *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the United States of America* **97,** 7539–44.

Miao, E.A., Scherer, C.A., Tsolis, R.M., Kingsley, R.A., Adams, L.G., Baumler, A.J. and Miller, S.I. (1999). *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Molecular Microbiology* **34,** 850–64.

Miller, R.V. (2001). Environmental bacteriophage-host interactions: factors contribution to natural transduction. *Antonie van Leeuwenhoek* **79,** 141–7.

Mirold, S., Ehrbar, K., Weissmuller, A., Prager, R., Tschape, H., Russmann, H. and Hardt, W.D. (2001a). *Salmonella* host cell invasion emerged by acquisition of a mosaic of separate genetic elements, including *Salmonella* pathogenicity island 1 (SPI1), SPI5, and sopE2. *Journal of Bacteriology* **183,** 2348–58.

Mirold, S., Rabsch, W., Tschape, H. and Hardt, W.D. (2001b). Transfer of the *Salmonella* type III effector *sopE* between unrelated phage families. *Journal of Molecular Biology* **312,** 7–16.

Molmeret, M., Alli, O.A., Zink, S., Flieger, A., Cianciotto, N.P. and Kwaik, Y.A. (2002). *IcmT* is essential for pore formation-mediated egress of *Legionella pneumophila* from mammalian and protozoan cells. *Infection & Immunity* **70,** 69–78.

Mooi, F.R., van Oirschot, H., Heuvelman, K., van der Heide, H.G., Gaastra, W. and Willems, R.J. (1998). Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infection & Immunity* **66,** 670–5.

Moriishi, K., Koura, M., Abe, N., Fujii, N., Fujinaga, Y., Inoue, K. and Ogumad, K. (1996a). Mosaic structures of neurotoxins produced from *Clostridium botulinum* types C and D organisms. *Biochimica et Biophysica Acta* **1307,** 123–6.

Moriishi, K., Koura, M., Fujii, N., Fujinaga, Y., Inoue, K., Syuto, B. and Oguma, K. (1996b). Molecular cloning of the gene encoding the mosaic neurotoxin, composed of parts of botulinum

neurotoxin types C1 and D, and PCR detection of this gene from *Clostridium botulinum* type C organisms. *Applied & Environmental Microbiology* **62,** 662–7.

Moriishi, K., Syuto, B., Saito, M., Oguma, K., Fujii, N., Abe, N. and Naiki, M. (1993). Two different types of ADP-ribosyltransferase C3 from *Clostridium botulinum* type D lysogenized organisms. *Infection & Immunity* **61,** 5309–14.

Muller, D., Hughes, C. and Goebel, W. (1983). Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli. Journal of Bacteriology* **153,** 846–51.

Muniesa, M., Serra-Moreno, R. and Jofre, J. (2004). Free Shiga toxin bacteriophages isolated from sewage showed diversity although the *stx* genes appeared conserved. *Environmental Microbiology* **6,** 716–25.

Musser, J.M., Hauser, A.R., Kim, M.H., Schlievert, P.M., Nelson, K. and Selander, R.K. (1991). *Streptococcus pyogenes* causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 2668–72.

Nakayama, K., Kanaya, S., Ohnishi, M., Terawaki, Y. and Hayashi, T. (1999). The complete nucleotide sequence of phi CTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa:* implications for phage evolution and horizontal gene transfer via bacteriophages. *Molecular Microbiology*. **31,** 399–419.

Narita, S., Kaneko, J., Chiba, J., Piemont, Y., Jarraud, S., Etienne, J. and Kamio, Y. (2001). Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus:* molecular analysis of a PVL-converting phage, phiSLT. *Gene* **268,** 195–206.

Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli. Microbiology Reviews* **11,** 142–201.

Newland, J.W. and Neill, R.J. (1988). DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. *Journal of Clinical Microbiology* **26,** 1292–7.

Nhieu, G.T. and Sansonetti, P.J. (1999). Mechanism of *Shigella* entry into epithelial cells. *Current Opinion in Microbiology* **2,** 51–5.

Nijsten, R., London, N., van den Bogaard, A. and Stobberingh, E. (1995). *In-vivo* transfer of resistance plasmids in rat, human, or pig-derived intestinal flora using a rat model. *Journal of Antimicrobial Chemotherapy* **36,** 975–85.

Normander, B., Christensen, B.B., Molin, S. and Kroer, N. (1998). Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*). *Applied & Environmental Microbiology* **64,** 1902–9.

Novichkov, P.S., Omelchenko, M.V., Gelfand, M.S., Mironov, A.A., Wolf, Y.I. and Koonin, E.V. (2004). Genome-wide molecular clock and horizontal gene transfer in bacterial evolution. *Journal of Bacteriology* **186,** 6575–85.

Novick, R.P. (2003). Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. *Plasmid* **49,** 93–105.

Okinaka, R., Cloud, K., Hampton, O., Hoffmaster, A., Hill, K., Keim, P., Koehler, T., Lamke, G., Kumano, S., Manter, D., Martinez, Y., Ricke, D., Svensson, R. and Jackson, P. (1999a). Sequence, assembly, and analysis of pXo1 and pXo2. *Journal of Applied Microbiology* **87,** 261–2.

Okinaka, R.T., Cloud, K., Hampton, O., Hoffmaster, A.R., Hill, K.K., Keim, P., Koehler, T.M., Lamke, G., Kumano, S., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, R. and Jackson, P.J. (1999b). Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *Journal of Bacteriology* **181,** 6509–15.

Omelchenko, M.V., Makarova, K.S., Wolf, Y.I., Rogozin, I.B. and Koonin, E.V. (2003). Evolution of mosaic operons by horizontal gene transfer and gene displacement *in situ*. *Genome Biology* **4,** R55.

Onderdonk, A.B., Lowe, B.R. and Bartlett, J.G. (1979). Effect of environmental stress on *Clostridium difficile* toxin levels during continuous cultivation. *Applied & Environmental Microbiology* **38,** 637–41.

Oswald, E., de Rycke, J., Lintermans, P., van Muylem, K., Mainil, J., Daube, G. and Pohl, P. (1991). Virulence factors associated with cytotoxic necrotizing factor type two in bovine diarrheic and septicemic strains of *Escherichia coli. Journal of Clinical Microbiology* **29,** 2522–7.

Oswald, E., Pohl, P., Jacquemin, E., Lintermans, P., Van Muylem, K., O'Brien, A.D. and Mainil, J. (1994a). Specific DNA probes to detect *Escherichia coli* strains producing cytotoxic necrotizing factor type 1 or type 2. *Journal of Medical Microbiology* **40,** 428–34.

Oswald, E., Sugai, M., Labigne, A., Wu, H.C., Fiorentini, C., Boquet, P. and O'Brien, A.D. (1994b). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proceedings of the National Academy of Sciences of the United States of America* **91,** 3814–8.

Pannucci, J., Okinaka, R.T., Sabin, R. and Kuske, C.R. (2002). *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. *Journal of Bacteriology* **184,** 134–41.

Pappenheimer, A.M., Jr. and Murphy, J.R. (1983). Studies on the molecular epidemiology of diphtheria. *Lancet* **2,** 923–6.

Parkhill, J., Sebaihia, M., Preston, A., Murphy, L.D., Thomson, N., Harris, D.E., Holden, M.T., Churcher, C.M., Bentley, S.D., Mungall, K.L., Cerdeno-Tarraga, A.M., Temple, L., James, K., Harris, B., Quail, M.A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A., Davis, P., Doggett, J., Feltwell, T., Goble, A., Hamlin, N., Hauser, H., Holroyd, S., Jagels, K., Leather, S., Moule, S., Norberczak, H., O'Neil, S., Ormond, D., Price, C., Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B.G. and Maskell, D.J. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica. Nature Genetics* **35,** 32–40.

Parsot, C. and Sansonetti, P.J. (1996). Invasion and the pathogenesis of *Shigella* infections. *Current Topics in Microbiology & Immunology* **209,** 25–42.

Paul, J.H., Sullivan, M.B., Segall, A.M. and Rohwer, F. (2002). Marine phage genomics. Comparative Biochemistry & Physiology. *Part B, Biochemistry & Molecular Biology* **133,** 463–76.

Pearson, G.D., Woods, A., Chiang, S.L. and Mekalanos, J.J. (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 3750–4.

Peres, S.Y., Marches, O., Daigle, F., Nougayrede, J.P., Herault, F., Tasca, C., De Rycke, J. and Oswald, E. (1997). A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. *Molecular Microbiology* **24,** 1095–107.

Popoff, M., Boquet, P., Gill, D.M. and Eklund, M.W. (1990). DNA sequence of exoenzyme C3, an ADP-ribosyltransferase encoded by *Clostridium botulinum* C and D phages. *Nucleic Acids Research* **18,** 1291.

Preston, A., Parkhill, J. and Maskell, D.J. (2004). The *Bordetellae*: lessons from genomics. Nature Reviews. *Microbiology* **2,** 379–90.

Price, L.B., Hugh-Jones, M., Jackson, P.J. and Keim, P. (1999). Genetic diversity in the protective antigen gene of *Bacillus anthracis. Journal of Bacteriology* **181,** 2358–62.

Prodinger, W.M., Fille, M., Bauernfeind, A., Stemplinger, I., Amann, S., Pfausler, B., Lass-Florl, C. and Dierich, M.P. (1996). Molecular epidemiology of Klebsiella pneumoniae producing SHV-5

beta-lactamase: parallel outbreaks due to multiple plasmid transfer. *Journal of Clinical Microbiology* **34,** 564–8.

Pullinger, G.D., Bevir, T. and Lax, A.J. (2004). The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. *Molecular Microbiology* **51,** 255–69.

Rappuoli, R., Gross, R. and Arico, B. (1987). Conversion from *Bordetella parapertussis* to *B pertussis*. *Lancet* **2,** 511.

Ratti, G., Rappuoli, R. and Giannini, G. (1983). The complete nucleotide sequence of the gene coding for diphtheria toxin in the corynephage omega (*tox+*) genome. *Nucleic Acids Research* **11,** 6589–95.

Reacher, M., Ramsay, M., White, J., De Zoysa, A., Efstratiou, A., Mann, G., Mackay, A. and George, R.C. (2000). Nontoxigenic *Corynebacterium diphtheriae*: an emerging pathogen in England and Wales? *Emerging Infectious Diseases* **6,** 640–5.

Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., Gill, S.R., Holtzapple, E.K., Okstad, O.A., Helgason, E., Rilstone, J., Wu, M., Kolonay, J.F., Beanan, M.J., Dodson, R.J., Brinkac, L.M., Gwinn, M., DeBoy, R.T., Madpu, R., Daugherty, S.C., Durkin, A.S., Haft, D.H., Nelson, W.C., Peterson, J.D., Pop, M., Khouri, H.M., Radune, D., Benton, J.L., Mahamoud, Y., Jiang, L., Hance, I.R., Weidman, J.F., Berry, K.J., Plaut, R.D., Wolf, A.M., Watkins, K.L., Nierman, W.C., Hazen, A., Cline, R., Redmond, C., Thwaite, J.E., White, O., Salzberg, S.L., Thomason, B., Friedlander, A.M., Koehler, T.M., Hanna, P.C., Kolsto, A.B. and Fraser, C.M. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423,** 81–6.

Ripp, S., Ogunseitan, O.A. and Miller, R.V. (1994). Transduction of a freshwater microbial community by a new *Pseudomonas aeruginosa* generalized transducing phage, UT1. *Molecular Ecology* **3,** 121–6.

Rodriguez Jovita, M., Collins, M.D. and East, A.K. (1998). Gene organization and sequence determination of the two botulinum neurotoxin gene clusters in *Clostridium botulinum* type A(B) strain NCTC 2916. *Current Microbiology* **36,** 226–31.

Sandstrom, J.P., Russell, J.A., White, J.P. and Moran, N.A. (2001). Independent origins and horizontal transfer of bacterial symbionts of aphids. *Molecular Ecology* **10,** 217–28.

Santos-Buelga, J.A., Collins, M.D. and East, A.K. (1998). Characterization of the genes encoding the botulinum neurotoxin complex in a strain of *Clostridium botulinum* producing type B and F neurotoxins. *Current Microbiology* **37,** 312–8.

Sato, H. and Frank, D.W. (2004). ExoU is a potent intracellular phospholipase. *Molecular Microbiology* **53,** 1279–90.

Schlimme, W., Baur, B., Hanselmann, K. and Jenni, B. (1995). An agarose slide method to follow the fate of bacteria within digestive vacuoles of protozoa. *FEMS Microbiology Letters* **133,** 169–73.

Schmidt, H., Kernbach, C. and Karch, H. (1996). Analysis of the EHEC *hly* operon and its location in the physical map of the large plasmid of enterohemorrhagic *Escherichia coli* O157:h7. *Microbiology* **142,** 907–14.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology & Molecular Biology Reviews* **62,** 775–806.

Scott, K.P. and Flint, H.J. (1995). Transfer of plasmids between strains of *Escherichia coli* under rumen conditions. *Journal of Applied Bacteriology* **78,** 189–93.

Sheahan, K.L., Cordero, C.L. and Satchell, K.J. (2004). Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *Proc Natl Acad Sci U S A* **101,** 9798–803.

Shoemaker, N.B., Vlamakis, H., Hayes, K. and Salyers, A.A. (2001). Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Applied & Environmental Microbiology* **67,** 561–8.

Sierig, G., Cywes, C., Wessels, M.R. and Ashbaugh, C.D. (2003). Cytotoxic effects of streptolysin O and streptolysin S enhance the virulence of poorly encapsulated group A streptococci. *Infection & Immunity* **71,** 446–55.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407,** 762–4.

Skogen, V., Cherkasova, V.V., Maksimova, N., Marston, C.K., Sjursen, H., Reeves, M.W., Olsvik, O. and Popovic, T. (2002). Molecular characterization of *Corynebacterium diphtheriae* isolates, Russia, 1957–1987. *Emerging Infectious Diseases* **8,** 516–8.

Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Ricklefs, S.M., Porcella, S.F., Parkins, L.D., Beres, S.B., Campbell, D.S., Smith, T.M., Zhang, Q., Kapur, V., Daly, J.A., Veasy, L.G. and Musser, J.M. (2002). Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 4668–73.

Solomon, E.B., Potenski, C.J. and Matthews, K.R. (2002a). Effect of irrigation method on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. *Journal of Food Protection* **65,** 673–6.

Solomon, E.B., Yaron, S. and Matthews, K.R. (2002b). Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Applied & Environmental Microbiology* **68,** 397–400.

Strauch, E., Lurz, R. and Beutin, L. (2001). Characterization of a Shiga toxin-encoding temperate bacteriophage of *Shigella sonnei*. *Infection & Immunity* **69,** 7588–95.

Strauch, E., Schaudinn, C. and Beutin, L. (2004). First-time isolation and characterization of a bacteriophage encoding the Shiga toxin 2c variant, which is globally spread in strains of *Escherichia coli* O157. *Infect Immun*. **72,** 7030–9.

Stroeher, U.H., Bode, L., Beutin, L. and Manning, P.A. (1993). Characterization and sequence of a 33-kDa enterohemolysin (Ehly 1)-associated protein in *Escherichia coli*. *Gene* **132,** 89–94.

Tauxe, R.V., Cavanagh, T.R. and Cohen, M.L. (1989). Interspecies gene transfer *in vivo* producing an outbreak of multiply resistant shigellosis. *Journal of Infectious Diseases* **160,** 1067–70.

Taylor, R.K., Miller, V.L., Furlong, D.B. and Mekalanos, J.J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences of the United States of America* **84,** 2833–7.

Teuber, M. (2001). Veterinary use and antibiotic resistance. *Current Opinion in Microbiology*, **4,** 493–9.

Thomas, D.J., Morgan, J.A., Whipps, J.M. and Saunders, J.R. (2001). Plasmid transfer between *Bacillus thuringiensis* subsp. israelensis strains in laboratory culture, river water, and dipteran larvae. *Applied & Environmental Microbiology* **67,** 330–8.

Toth, I., Oswald, E., Mainil, J.G., Awad-Masalmeh, M. and Nagy, B. (2000). Characterization of intestinal *cnf1+ Escherichia coli* from weaned pigs. *International Journal of Medical Microbiology* **290,** 539–42.

Tyler, J.S., Mills, M.J. and Friedman, D.I. (2004). The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. *Journal Bacteriology* **186,** 7670–9.

Unkmeir, A. and Schmidt, H. (2000). Structural analysis of phage-borne stx genes and their flanking sequences in shiga toxin-producing *Escherichia* coli and *Shigella dysenteriae* type 1 strains. *Infection & Immunity* **68,** 4856–64.

van der Zee, A., Groenendijk, H., Peeters, M. and Mooi, F.R. (1996a). The differentiation of *Bordetella parapertussis* and *Bordetella bronchiseptica* from humans and animals as determined by DNA polymorphism mediated by two different insertion sequence elements suggests their phylogenetic relationship. *International Journal of Systematic Bacteriology* **46,** 640–7.

van der Zee, A., Vernooij, S., Peeters, M., van Embden, J. and Mooi, F.R. (1996b). Dynamics of the population structure of *Bordetella pertussis* as measured by IS1002-associated RFLP: comparison of pre- and post-vaccination strains and global distribution. *Microbiology* **142,** 3479–85.

Vavre, F., Fleury, F., Lepetit, D., Fouillet, P. and Bouletreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molecular Biology & Evolution* **16,** 1711–23.

von Hunolstein, C., Alfarone, G., Scopetti, F., Pataracchia, M., La Valle, R., Franchi, F., Pacciani, L., Manera, A., Giammanco, A., Farinelli, S., Engler, K., De Zoysa, A. and Efstratiou, A. (2003). Molecular epidemiology and characteristics of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains isolated in Italy during the 1990s. *Journal of Medical Microbiology* **52,** 181–8.

Wagner, P.L., Livny, J., Neely, M.N., Acheson, D.W., Friedman, D.I. and Waldor, M.K. (2002). Bacteriophage control of Shiga toxin 1 production and release by *Escherichia coli*. *Molecular Microbiology* **44,** 957–70.

Wagner, P.L., Neely, M.N., Zhang, X., Acheson, D.W., Waldor, M.K. and Friedman, D.I. (2001). Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. *Journal of Bacteriology* **183,** 2081–5.

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272,** 1910–4.

Walker, K.E. and Weiss, A.A. (1994). Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. *Infection & Immunity* **62,** 3817–28.

Wallis, T.S. and Galyov, E.E. (2000). Molecular basis of Salmonella-induced enteritis. *Molecular Microbiology* **36,** 997–1005.

Watanabe, K. and Sato, M. (1998). Plasmid-mediated gene transfer between insect-resident bacteria, *Enterobacter cloacae*, and plant-epiphytic bacteria, *Erwinia herbicola*, in guts of silkworm larvae. *Current Microbiology* **37,** 352–5.

Watnick, P.I., Fullner, K.J. and Kolter, R. (1999). A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *Journal of Bacteriology* **181,** 3606–9.

Weber, C., Boursaux-Eude, C., Coralie, G., Caro, V. and Guiso, N. (2001). Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. *Journal of Clinical Microbiology* **39,** 4396–403.

Wernegreen, J.J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nature Reviews Genetics* **3,** 850–61.

Wernegreen, J.J., Degnan, P.H., Lazarus, A.B., Palacios, C. and Bordenstein, S.R. (2003). Genome evolution in an insect cell: distinct features of an ant-bacterial partnership. *Biological Bulletin* **204,** 221–31.

Werren, J.H., Zhang, W. and Guo, L.R. (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proceedings of the Royal Society of London -Series B: Biological Sciences* **261,** 55–63.

Wilson, A.P. (1995). The return of *Corynebacterium diphtheriae:* the rise of non-toxigenic strains. *Journal of Hospital Infection* **30** Suppl, 306–12.

Winstanley, C. and Hart, C.A. (2001). Type III secretion systems and pathogenicity islands. *Journal of Medical Microbiology* **50,** 116–26.

Wolfgang, M.C., Kulasekara, B.R., Liang, X., Boyd, D., Wu, K., Yang, Q., Miyada, C.G. and Lory, S. (2003). Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **100,** 8484–9.

Yahr, T.L., Goranson, J. and Frank, D.W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. Molecular Microbiology **22,** 991–1003.

Yahr, T.L., Vallis, A.J., Hancock, M.K., Barbieri, J.T. and Frank, D.W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *National Academy of Sciences of the United States of America* **95,** 13899–904.

Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M., Nakayama, K., Yamada, S., Komatsuzawa, H. and Sugai, M. (2000). Phage conversion of exfoliative toxin A production in Staphylococcus aureus. *Molecular Microbiology* **38,** 694–705.

Yamaguchi, T., Hayashi, T., Takami, H., Ohnishi, M., Murata, T., Nakayama, K., Asakawa, K., Ohara, M., Komatsuzawa, H. and Sugai, M. (2001). Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infection & Immunity*. **69,** 7760–71.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M. and Sugai, M. (2002). Identification of the *Staphylococcus aureus* etd pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infection & Immunity* **70,** 5835–45.

Yoon, S.S., Hennigan, R.F., Hilliard, G.M., Ochsner, U.A., Parvatiyar, K., Kamani, M.C., Allen, H.L., DeKievit, T.R., Gardner, P.R., Schwab, U., Rowe, J.J., Iglewski, B.H., McDermott, T.R., Mason, R.P., Wozniak, D.J., Hancock, R.E., Parsek, M.R., Noah, T.L., Boucher, R.C. and Hassett, D.J. (2002). *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Developmental Cell* **3,** 593–603.

Yu, C.E. and Ferretti, J.J. (1989). Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (*speA*) in clinical *Streptococcus pyogenes* strains. *Infection & Immunity* **57,** 3715–9.

Yu, C.E. and Ferretti, J.J. (1991a). Frequency of the erythrogenic toxin B and C genes (*speB* and *speC*) among clinical isolates of group A streptococci. *Infection & Immunity* **59,** 211–5.

Yu, C.E. and Ferretti, J.J. (1991b). Molecular characterization of new group A streptococcal bacteriophages containing the gene for streptococcal erythrogenic toxin A (*speA*). *Molecular & General Genetics* **231,** 161–8.

Zhang, L., Murphy, P.J., Kerr, A. and Tate, M.E. (1993). *Agrobacterium* conjugation and gene regulation by N-acyl-L-homoserine lactones. *Nature* **362,** 446–8.

Zhang, S., Santos, R.L., Tsolis, R.M., Mirold, S., Hardt, W.D., Adams, L.G. and Baumler, A.J. (2002). Phage mediated horizontal transfer of the *sopE1* gene increases enteropathogenicity of *Salmonella enterica* serotype Typhimurium for calves. *FEMS Microbiology Letters* **217,** 243–7.

Zhou, D. and Galan, J. (2001). *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes & Infection* **3,** 1293–8.

Zhou, Y., Sugiyama, H. and Johnson, E.A. (1993). Transfer of neurotoxigenicity from *Clostridium butyricum* to a nontoxigenic *Clostridium botulinum* type E-like strain. *Applied & Environmental Microbiology* **59,** 3825–31.

Zhou, Y., Sugiyama, H., Nakano, H. and Johnson, E.A. (1995). The genes for the *Clostridium botulinum* type G toxin complex are on a plasmid. *Infection & Immunity* **63,** 2087–91.

CHAPTER

# 3

# Mobile genetic elements and pathogenicity islands encoding bacterial toxins

*Ulrich Dobrindt and Jörg Hacker*

## INTRODUCTION: THE GENOME STRUCTURE OF PROKARYOTES

Pathogenicity correlates with the expression of disease-related factors present in pathogenic bacteria but usually absent from non-pathogenic bacteria. The analyses of complete prokaryotic genome sequences have shown that the bacterial genome size and organization is considerably variable. Different numbers of circular or linear chromosomes, extrachromosomal linear or circular replicons, as well as different combinations thereof, exist in bacteria (Bentley and Parkhill, 2004). The bacterial chromosome is considered to be composed of a conserved "core" gene pool harboring genetic information required for essential cellular functions and of a "flexible" gene pool. The latter encodes additional traits that contribute to the adaptation of microbes under certain circumstances, e.g., resistance to antibiotics, production of toxic compounds, as well as other virulence factors. The chromosomal organization of the "core" regions is similar in closely related species and is not transferable *per se*. Genes located within this chromosomal backbone exhibit a relatively homogenous G+C content and a specific codon usage (Sharp, 1991). In contrast, the "flexible" gene pool consists of variable chromosomal regions which may be beneficial under certain circumstances, e.g., bacterial toxins and other virulence factors. This gene pool includes mobile and accessory genetic elements such as bacteriophages, plasmids, genomic islands, IS elements, transposons, and integrons.

Genomic islands (GEIs) represent a group of distinct genetic entities. Depending on the functions and their role for a specific lifestyle of a bacterium, GEIs may be called pathogenicity, symbiosis, fitness, metabolic, or resistance islands (Hentschel and Hacker, 2001; Hacker and Carniel, 2001; Hacker and Kaper, 2002). Furthermore, the presence of identical genes in pathogenic and non-pathogenic variants of one species, e.g., in extraintestinal pathogenic and commensal *E. coli*, implies that some of these encoded factors rather contribute to general adaptability, fitness, and competitiveness than to particular virulence traits (Grozdanov *et al*., 2004; Dobrindt *et al*., 2004). Other accessory components have been described like the transposable Insertion Sequence (IS) elements and transposons (Deonier, 1996). They are restricted to moving themselves and sometimes additional sequences, by recombination events from one site of their genome to other sites of the same genome (Berg, 1989). In addition to the chromosome(s), many prokaryotes can contain genetic information on plasmids, which are extrachromosomal DNA elements with the ability of autonomous replication. The genetic information encoded by plasmids can contain valuable genes, which may be beneficial under certain conditions, such as toxin genes, but also resistance determinants and genes coding for specific metabolic properties. Many different plasmid types can be transferred between

bacteria. Some have the ability to become integrated into the chromosome, thereby losing control of their own replication (Hardy, 1986; Lüneberg *et al*., 2001). Other genetic elements frequently found in association with the genome are integrons, retrophages, and lysogenic bacteriophages. Transferable genetic elements, such as bacteriophages and plasmids, can function as vehicles laterally transporting genetic information, thus playing an important role in bacterial evolution. Bacteriophages, as viruses that infect prokaryotes, can genetically modify their host in becoming part of the genome. They may carry genes that bring about new functions or modify existing ones. Interestingly, toxin-specific genes are often located on bacteriophage genomes (Travisano and Inouye, 1995; Mazel *et al*., 1998; Rowe-Magnus *et al*., 2001; Brüssow *et al*., 2004).

With respect to the above-mentioned structure of bacterial chromosomes and the different classes of mobile elements frequently found in prokaryotic genomes, it can be concluded that the genome of prokaryotes constantly undergoes structural variations due to the potential of mobile elements to integrate into different sites of the chromosome and to promote chromosomal rearrangements via recombinational mechanisms. Genome plasticity, i.e., the frequently occurring acquisition or loss of genetic information, is of great importance for adaptive evolution of disease-causing bacteria as simultaneous acquisition of many genes by HGT allows the inheritance of complex disease-related characteristics, including protein toxins, in a single step (Ochman *et al*., 2000; Ochman and Moran, 2001; Hacker *et al*., 2003).

The accumulation of prokaryotic gene and genome sequences has shed light on many aspects of microbiology. The current knowledge of more than 200 complete microbial genome sequences (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html) has proven particularly useful for comparative studies between different bacterial species and the evolutionary processes that drive their differentiation. Comparative genomics reveals that a striking correlation exists between the distribution of protein toxins and movable genetic elements in bacteria. Indeed, a great number of bacterial protein toxin determinants are associated with plasmids, bacteriophages, or pathogenicity islands. Together with gene loss and other genomic alterations, gene acquisition plays an important role for adaptive evolution of prokaryotes. Considerable insight has been gained into the contribution of mobile and accessory genetic elements to these processes. In this review, we will show that there is a structural and functional interdependence between many bacterial protein toxin genes and mobile and accessory genetic elements that promotes adaptive evolution of pathogenic bacteria.

## PROTEIN TOXINS ENCODED BY MOBILE GENETIC ELEMENTS

### Protein toxins encoded by plasmids

Many bacterial pathogens harbor plasmids carrying protein toxin determinants. They frequently contribute to specific combinations of virulence factors present in these strains. This argues for a co-evolution of specific factors in different pathotypes. Some of these plasmids have been shown to integrate into the chromosome (Zagaglia *et al*., 1991; Colonna *et al*., 1995). Important plasmid-encoded protein toxins are given in Table 3.1.

#### *Gram-negative bacteria*

Intestinal *E. coli* bacteria may cause different types of diarrheal diseases. The differences in the clinical pictures reflect the different pathotypes of intestinal *E. coli*, such as ETEC (enterotoxigenic *E. coli*), EPEC (enteropathogenic *E. coli*), and others (see Table 3.1). A main feature of the different intestinal *E. coli* pathotypes is the presence of pathotype-specific plasmids, which often encode protein toxins. Enterotoxigenic *E. coli* (ETEC) strains cause diarrhea through the action of two different plasmid-encoded types of enterotoxins, the heat-labile enterotoxin (LT) and the heat-stable enterotoxin (ST). These strains usually contain the determinant for an LT only, an ST only, or both toxin types (Gyles *et al*., 1974; Levine, 1987; Gyles, 1992). Heat-labile toxins (LTs) are closely related to the cholera toxin of *Vibrio cholerae* (Spangler, 1992; Sixma *et al*., 1993). Two unrelated classes of plasmid-encoded heat-stable toxins without sequence homology exist (STa and STb). The STb encoding gene (*estB*) is found on heterogenous plasmids, which may also contain other properties (Harnett and Gyles, 1985; Echeverria *et al*., 1985; Dubreuil, 1997).

The low-molecular-weight ST called *EAST1 of enteroaggregative E. coli* (EAEC) strains (Savarino *et al*., 1991, 1993) exhibits 50% protein identity to STa and is encoded on so-called EAF-plasmids, which range from 50–70 MDa in size (Nataro *et al*., 1987). This enterotoxin can also be expressed in addition to STa from some ETEC, EHEC, and many EPEC strains as well (Nataro *et al*., 1987; Savarino *et al*., 1996; Yamamoto and Echeverria, 1996).

Enteroinvasive *E. coli* produce an enterotoxin that is also expressed by *Shigella flexneri*. Therefore, it has been designated ShET2 (*Shigella* enterotoxin 2) as well. The toxin-encoding gene (*sen*) is located on the 140-MDa pInv-plasmid (Nataro *et al*., 1995; Sears and Kaper, 1996).

**TABLE 3.1** Protein toxins encoded by bacterial plasmids

| Organism | Pathotype[1] | Plasmid-encoded property | Gene symbol | Other plasmid-encoded factors |
|---|---|---|---|---|
| *Escherichia coli* | ETEC | Heat-labile enterotoxin (LT) | *elt*, *etx* | ST, CFAs, drug resistance, colicins |
| | ETEC | Heat-stable enterotoxin (ST)[2] | *est* | ST, LT, CFAs, drug resistance, colicins |
| | EAEC, ETEC, EHEC, EPEC | EAST1 | *ast* | Type IV adhesin "bundle forming pili" |
| | ExPEC | Alpha-hemolysin (Hly) | *hly* | - |
| | EHEC | Enterohemolysin (Ehx) | *ehx* | - |
| | ExPEC, ETEC | Cytotoxic necrotizing factor 2 (CNF2) | *cnf*2 | F17-, AFA/Dr adhesins, cytolethal distending toxin (CDT) |
| | EIEC | EIEC enterotoxin ( = *Shigella* enterotoxin 2) | *sen* | Genes required for invasion, Ipa proteins, type III secretion system |
| *Yersinia spp*. | | Yop proteins | *yop* | Genes required for invasion, Yop proteins, type III secretion system |
| *Shigella spp*. | | *Shigella* enterotoxin 2 (shET2-2, OspD2) | *senA* | Genes required for invasion, Ipa proteins, type III secretion system |
| | | *Shigella* enterotoxin SenA (OspD3) | | |
| *Enterococcus faecalis* | | Cytolysin | *cyl* | - |
| *Staphylococcus aureus* | | Enterotoxin type D and J | *entD, -J* | Penicillin and cadmium resistance |
| | | Exfoliative toxin B | *etb* | Cadmium resistance, bacteriocin |
| | | ADP-ribosyltransferase EDIN-C | *edin-C* | see above |
| *Clostridium tetani* | | Tetanus neurotoxin (TeTx) | *tetX* | Collagenase (ColT) |
| *Clostridium botulinum* | | Botulinum neurotoxin type G (BoNT/G) | *botG* | Bacteriocin (Boticin G) |
| *Bacillus anthracis* | | Anthrax toxin (lethal factor, edema factor, protective antigen) | *lef*, *cya*, *pag* | - |

[1]EAEC, enteroaggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*
[2]Genes encoding for STa (*estA*) and STb (*estB*) have been found on Tn1681 (So and McCarthy, 1980) and Tn4521(Lee et al., 1985; Hu et al., 1987; Hu and Lee, 1988), respectively.

ETEC strains and extraintestinal *E. coli* from animals often carry the structural gene for CNF2 (*cnf*2) on the F-like Vir-plasmid (Oswald and De Rycke, 1990; Oswald *et al*., 1994). The Vir-plasmid can also contain sequences that are homologous to adhesins as well as cytolethal distending toxin (CDT) determinants (Mainil *et al*., 1997; Peres *et al*., 1997). Interestingly, *cnf1*, which is a homologous gene of *cnf*2, is located on pathogenicity islands of uropathogenic *E. coli* strains (Blum *et al*., 1995; Donnenberg and Welch, 1995; Swenson *et al*., 1996).

Extraintestinal *E. coli*, a major cause of urinary tract infections (UTI), sepsis, and newborn meningitis (NBM) in humans, often carry pathogenicity islands (PAIs) that contain the α-hemolysin gene cluster (*hly*) (Hacker and Hughes, 1985). Some *E. coli* strains contain *hly* determinants on plasmids, which are heterogenous in size (50 to 160 kb), conjugational behavior, and incompatibility group (De la Cruz *et al*., 1980). The plasmid- and chromosomally encoded *hly* determinants show a high overall homology (De la Cruz *et al*., 1980; Müller *et al*., 1983). Nevertheless, the sequences of the upstream regions differ significantly (Knapp *et al*., 1985; Hess *et al*., 1986). The enterohemolysin determinant (*ehxABD*) of enterohemorrhagic *E. coli* strains is encoded on plasmids (pO157) that range in size from 93–104 kb (Schmidt *et al*., 1995, 1996). Although this toxin has a high overall similarity to α-hemolysin, the amino- and carboxy-termini of both proteins are different (Schmidt and Karch, 1996; Bauer and Welch, 1996).

Virulence plasmids of pathogenic *Yersinia*, *Shigella*, and *Salmonella* species all have in common the fact that they lack genes encoding conventional protein toxins. The *Yersinia* and *Shigella* virulence plasmids code for a conserved type III protein secretion system (Lindler *et al*., 1998; Buchrieser *et al*., 2000). All pathogenic species of *Yersinia* (*Y. pestis*, *Y. pseudotuberculosis*, and

*Y. enterocolitica*) harbor a virulence plasmid (pYV) of about 70 kb in size. This plasmid encodes the Yop virulon that organizes the secretion of several proteins termed Yops (*Yersinia* outer proteins), which are essential for virulence (Portnoy and Falkow, 1981; Galyov *et al*., 1993; Straley *et al*., 1993). Some of the Yops are considered as "unconventional toxins," i.e., the cytotoxin YopE, YopH, and YopO (Bliska *et al*., 1992), as well as YopB which shows sequence similarity to contact hemolysins, like IpaB of *Shigella* species (Beuscher *et al*., 1995). All virulent *Shigellae* and enteroinvasive *E. coli* strains harbor a 220-kb pInv plasmid that encodes all genes which are essential for epithelial cell invasion (Parsot and Sansonetti, 1996). The effectors of the invasion process, termed *invasion plasmid antigens* (Ipa), are encoded on this plasmid (Venkatesan *et al*., 1988; Sasakawa *et al*., 1989; Venkatesan *et al*., 1991), as well as the invasin IpaB, which has homology to pore-forming toxins (Ménard *et al*., 1994; Beuscher *et al*., 1995). In addition, the invasion plasmid contains the *sen* gene encoding the *Shigella* enterotoxin 2, which is also called *EIEC enterotoxin*. The *sen* gene from *Shigella flexneri* 2a and EIEC share 99% identity (Nataro *et al*., 1995).

### *Gram-positive bacteria*

The *Clostridium tetani* structural gene encoding the tetanus neurotoxin (TeTx) is located on a 74-kb plasmid (e.g., pCL1, pE88) (Laird *et al*., 1980; Finn *et al*., 1984; Brüggemann *et al*., 2003). All *Cl. botulinum* type G strains investigated, contain an 81-MDa plasmid (Strom *et al*., 1984), which presumably encodes either the structural gene (*botG*) of the botulinum neurotoxin type G (BoNT/G) or regulatory genes important for BoNT/G expression (Eklund *et al*., 1988; Minton, 1995).

Virulent *Bacillus anthracis* strains contain two large plasmids, pXO1 and pXO2, which are both required for virulence (Kaspar and Robertson, 1987). The genes for the three-component anthrax exotoxin are located on pXO1 (Mikesell *et al*., 1983; Thorne, 1985), whereas plasmid pXO2 contains genes coding for another virulence factor of *B. anthracis*, the D-glutamic acid–composed capsule (Green *et al*., 1985; Uchida *et al*., 1985). The "pathogenicity region" of pXO1 is flanked by mobile genetic elements that are supposed to be involved in acquisition of this region by pXO1 (Okinaka *et al*., 1999). Comparative analysis of the complete genome sequence of *B. anthracis* strain Ames and of its non-pathogenic relative *B. cereus* strain ATCC10987 demonstrated that pXO1 (~182 kb) is similar to plasmid pBc10987 (~208 kb) of *B. cereus*. Roughly 65% of the encoded proteins were homologous and about 50% of them were in a syntenic location. Interestingly, the region of pXO1 comprising the genes that code for the lethal factor, protective antigen, edema factor, and the transcriptional regulator AtxA is absent from pBc10987. Instead, a *B. cereus*-specific region can be found that is not flanked by mobile genetic elements (Rasko *et al*., 2004). Similar observations have been made by comparative genome hybridization of 19 different isolates of the *B. cereus*/*B. thuringiensis* group to a DNA array based on the *B. anthracis* strain Ames genome sequence. The results obtained indicate that pXO1 homologues that lack the pathogenicity region were detectable in 50% of the tested *B. cereus*/*B. thuringiensis* strains (Read *et al*., 2003). These data underline that variability in plasmid gene content and the acquisition of plasmid-encoded virulence factors play an important role in evolution of pathogenic bacteria.

Cytolytic strains of *Enterococcus faecalis* produce a cytolysin that shows a homology to lantibiotics. The cytolysin determinant (*cyl*) consists of six tandemly arranged genes. These are encoded in one operon on large ( 60 kb), transmissible, and pheromone-responsive plasmids (Jett *et al*., 1994; Gilmore *et al*., 1994). Cytolysin determinants have also been located on the chromosome of *Enterococcus* (Ike and Clewell, 1992; Shankar *et al*., 2002).

The structural gene (*entD*) coding for the staphylococcal enterotoxin D (SED) has been located on the 27.6-kb penicillinase plasmid pIB485 (Bayles and Iandolo, 1989). The two exfoliative toxin determinants responsible for the staphylococcal scalded skin syndrome have been assigned to different genetic loci. The gene for exfoliative toxin A (*eta*) has been mapped on the chromosome and that for the exfoliative toxin B (*etb*) on the plasmid pRW0019 (Warren *et al*., 1975; Jackson and Iandolo, 1985).

## Protein toxins encoded by bacteriophage

Bacteriophages encode a variety of toxin genes of pathogenic bacteria. The toxin genes are frequently located next to the bacteriophage attachment site, which argues for acquisition by mechanism of transduction. The bacteriophage-encoded toxins are given in Table 3.2.

### *Gram-negative bacteria*

Full virulence of *Vibrio cholerae* depends on two coordinately expressed factors: the cholera toxin (CT) and toxin-co-regulated pili (TCP). The CT encoding genes (*ctxAB*) are located on the CTX element, which ranges from 7 to 9.7 kb in size and occurs frequently in multiple, tandemly arranged copies. This element has been identified as a lysogenic filamentous bacteriophage designated CTXϕ (Mekalanos, 1983; Waldor and Mekalanos, 1996) and is restricted to toxigenic strains

**TABLE 3.2** Protein toxins encoded by bacteriophages

| Host organism | Bacteriophage-encoded toxin | Gene symbol | Phage designation |
|---|---|---|---|
| *Vibrio cholerae* | Cholera toxin (CT) | *ctx* | CTXϕ |
| *E. coli* (EHEC) | Shiga toxin (Stx) | *stx* | H19, 933 |
| *E. coli* | Enterohaemolysin | *hly*2 | ϕFC3208 |
| *E. coli* | Cytolethal distending toxin | *cdt* | – |
| *Pseudomonas aeruginosa* | Cytotoxin (CTX) | *ctx* | ϕCTX[1] |
| *Pasteurella multocida* | *Pasteurella multocida* toxin (PMT) | *toxA* | – |
| *Corynebacterium diphtheriae* | Diphtheria toxin (DT) | *tox* | β, ω[2] |
| *Clostridium botulinum* | Botulinum neurotoxin type C1 and D (BoNT/C1, BoNT/D) | *botC, botD* | C1 |
| *Streptococcus pyogenes* | Pyrogenic exotoxins A, C,G, H, I, M, L, K | *speA, -C, -I, -H, -M, -L, -K* | SPE-phage, CS112, T12[3] 8232.1, 315.5, 370.1, 370.2, 8232.3, 315.4 |
| | Streptococcal superantigen | *ssa* | 315.2 |
| *Staphylococcus aureus* | Enterotoxin A | *entA* | PS42D, ϕ13 |
| | Enterotoxin A | *sea* | ϕMu50A |
| | Enterotoxin G | *seg* | ϕSa3ms |
| | Enterotoxin K | *sek* | ϕSa3ms |
| | Enterotoxin P | *sep* | ϕN315 |
| | Exfoliative toxin A | *eta* | ϕETA |
| | Pantone-Valentine leukocidin | *lukSF*-PV | ϕPVL |
| | Staphylokinase | *sak* | ϕ13, ϕSa3ms |

[1]The chromosomal attachment site (*attB*) of ϕCTX has been mapped to the 3′-end of a $tRNA^{Ser}$-encoding gene (Hayashi et al., 1993).
[2]The chromosomal attachment sites (*attB1* and *attB2*) of β and ω overlap with a duplicate tRNA gene encoding the $tRNA_2^{Arg}$ (Ratti et al., 1997).
[3]The chromosomal attachment site (*attB*) of T12 has been mapped to the 3′-end of a $tRNA^{Ser}$-encoding gene (McShan et al., 1997).

(Miller and Mekalanos, 1984; Kovach *et al*., 1996). Structurally, the CTX element resembles a compound transposon (Pearson *et al*., 1993). The core region of the CTX element encodes several toxins, such as CT (*ctxAB*), zonula occludens toxin (*zot*), and the accessory cholera toxin (*ace*). The complete CTX element is self-transmissible and can replicate as a plasmid as well as lead to the production of extracellular virions (Waldor and Mekalanos, 1996). Transmission of CTXϕ requires the expression of TCP pili. The *tcp* genes are located on a separate pathogenicity island (Kovach *et al*., 1996; Faruque *et al*., 2003).

Shiga toxin (Vero toxin) is the major virulence factor of enterohemorrhagic *E. coli* strains. Two immunologically non-cross-reactive groups of Stx can be distinguished (Stx1 and Stx2). One EHEC strain expresses Stx1 only, Stx2 only, both toxin types, or multiple forms of Stx2 (O'Brien *et al*., 1992; Nataro and Kaper, 1998). The Stx1 of EHEC is essentially identical to Shiga toxin from *Shigella dysenteriae* (Takeda, 1995). Sequence variation exists among the members of the Stx1 and Stx2 groups (Jackson *et al*., 1987; O'Brien *et al*., 1992; Asakura *et al*., 2001; Zhang *et al*., 2002; Friedrich *et al*., 2002). The identically organized structural genes for Stx1 and Stx2 are located on lysogenic lambdoid phages, whereas those for Stx2v are encoded on the chromosome (Marques *et al*., 1987; Herold *et al*., 2004). Several variant Stx genes from human Shiga toxin–producing *E. coli* strains do not appear to be phage-encoded as well, but they may be carried on defective bacteriophages (Paton *et al*., 1992, 1993). Some *Citrobacter freundii* and *Enterobacter spp*. strains contain a *stx2* gene, which is homologous to that of *E. coli* (Schmidt *et al*., 1993).

The mitogenic *Pasteurella multocida* toxin PMT is also an example of a bacteriophage-encoded toxin. The coding gene *toxA* is located within a prophage genome that is chromosomally inserted at a tRNA-Leu gene in *P. multocida* strain LFB3 (Pullinger *et al*., 2004).

Certain *Pseudomonas aeruginosa* strains produce a cytotoxin (Ctx). The corresponding structural gene (*ctx*) is carried by a temperate bacteriophage (ϕCTX), which is able to convert non-toxigenic into toxigenic strains. The chromosomal attachment site (*attB*) of ϕCTX has been mapped to the 3′-end of the $tRNA^{Ser}$ (Hayashi *et al*., 1993; Nakayama *et al*., 1999).

### *Gram-positive bacteria*

The determinant encoding diphtheria toxin (*tox*) is part of the genome of corynephage β, which converts non-toxigenic *C. diphtheriae* strains into toxinogenic strains (Barksdale and Pappenheimer, 1954; Collier, 1982). Various different corynephages are known, including some non-converting phages. The corynephages ω and

β are able to form polylysogens by inserting into two different bacterial attachment sites, *attB1* and *attB2*. As a result, the level of toxigenicity is increased as the *tox* gene dose also influences the toxin production (Rappuoli and Ratti, 1984). Both attachment sites overlap with a duplicate tRNA gene encoding the $tRNA_2^{Arg}$ (Ratti *et al*., 1997).

Although many different *Cl. botulinum* strains carry bacteriophages, only in the case of the structural genes of BoNT/C1 and BoNT/D (*botC* and *botD*) has it been proven that they are part of the genomes of bacteriophages (Inoue and Iida, 1970; Eklund *et al*., 1971).

Streptococcal pyrogenic exotoxins and related exotoxins of *Staphylococcus aureus* belong to a family of molecules also designated pyrogenic toxin superantigens (PTSAgs) (Marrack and Kappler, 1990). Eight different types of SAgs have been identified for group A streptococci (GAS). The genome sequences of five GAS strains have been analyzed and indicate that each strain harbors four to eight prophages or prophage-like elements, most of which encode PTSAgs. The structural genes of the streptococcal pyrogenic exotoxins (SPE) type A, C, H, I, L, M, and K, as well as that of the streptococcal superantigen Ssa, have been shown to be phage-encoded. The prophages, which represent a small fraction of the complete GAS genome, account for up to 74% of the gene content variation among different strains. Only three out of 21 GAS prophage elements currently sequenced do not encode virulence-associated factors. Accordingly, they have been important for evolution and genome diversification of GAS genomes and may also be responsible for the spread of related toxins among related species (Weeks and Ferretti, 1984; McShan and Ferretti, 1997; Feretti *et al*., 2001; Smoot *et al*., 2002; Beres *et al*., 2002; Nakagawa *et al*., 2003; Banks *et al*., 2004). The description of the *S. dysgalactiae* mitogen (SDM) underlines that similar toxins exist in other streptococci as well (Miyoshi-Akiyama *et al*., 2003). Furthermore, two pyrogenic superantigens have been detected in *S. equi* (SePE-H and SePE-I), which are closely related to SPE H and I of *S. pyogenes* and the *Staphylococcus aureus* superantigens SEL, SEI, and SEM, respectively (Artiushin *et al*., 2002).

The staphylococcal enterotoxins A–Q also belong to the PTSAg family. The majority of them are encoded by (formerly) mobile genetic elements, but only the *sea*, *see*, *seg*, and *sek* genes are located on bacteriophages. The structural gene (*sak*) for the staphylokinase of *Staphylococcus aureus* is encoded by a bacteriophage (Sako and Tsuchida, 1983), as well as those coding for the exfoliating toxin A and Panton-Valentine leukocidin (Betley and Mekalanos, 1985; Yamaguchi *et al*., 2000; Kaneko *et al*., 1998; Narita *et al*., 2001; Sumby and Waldor, 2003).

## Protein toxin genes and other mobile genetic elements

Plasmids and bacteriophages are elements that increase the genetic flexibility of bacteria. They contribute to the evolution of pathogens upon horizontal gene transfer followed by integration into the chromosome. The fact that toxin genes have the capacity to spread among bacterial strains and even between species is underlined by the occurrence of identical toxin determinants or those with similar functions on different genetic elements, such as chromosomes, phages, and plasmids (see also Table 3.3). Thus, the

**TABLE 3.3** The location of protein toxin encoding genes and their homologues[1]

| Toxin | Location | Organism | Homologue | Location | Organism |
|---|---|---|---|---|---|
| Cholera toxin (CT) | Phage | *V. cholerae* | Heat-labile enterotoxin (LT) | Plasmid, chromosome | *E. coli* |
| α-Hemolysin | Chromosome, PAI | *E. coli* | α-Hemolysin | Plasmid | *E. coli* |
| Cytolysin | Chromosome, PAI | *E. faecalis* | Cytolysin | Plasmid | *E. faecalis* |
| CNF 1 | Chromosome, PAI | *E. coli* | CNF 2 | Plasmid | *E. coli* |
| Vat (autotransporter serine protease) | Chromosome, PAI | *E. coli* | Autotransporters, serine proteases | Plasmid, chromosome, PAIs | *E. coli* |
| Toxin complex | Chromosome, PAI | *P. luminescens* | Toxin complex | Plasmid | *S. entomophila* |
| TeTx | Plasmid | *Cl. tetani* | BoNT/C, BoNT/D | Phage | *Cl. botulinum* |
| BoNT/G | Plasmid | *Cl. botulinum* | BoNT/A, BoNT/F | Chromosome ? | *Cl. botulinum* |
| | | | BoNT/E | Plasmid, Phage ? | *Cl. butyricum* |
| ETA | Phage | *S. aureus* | ETD | Chromosome, PAI | *S. aureus* |
| ETB | Plasmid | *S. aureus* | ETA | Phage | *S. aureus* |
| | | | ETD | Chromosome, PAI | |
| EDIN-C | Plasmid | *S. aureus* | EDIN-B | Chromosome, PAI | *S. aureus* |
| SEA | Phage | *S. aureus* | SED | Plasmid | *S. aureus* |

[1]Not all homologues are listed for each toxin.

*E. coli* heat-labile enterotoxin (LT) genes (*elt*, *etx*) are located on plasmids (Nataro and Kaper, 1998), while the related cholera toxin (CT) structural gene (*ctxAB*) of toxigenic *V. cholerae* strains is phage-associated (Waldor and Mekalanos, 1996). A similar situation is reported for the Shiga toxin structural genes of *Sh. dysenteriae* (chromosomally encoded) (Hale, 1991) and *E. coli* (phage-encoded). The genes encoding α-hemolysin and CNF- toxins of pathogenic *E. coli* may be located either on plasmids (Waalwijk *et al*., 1984; Hess *et al*., 1986; Oswald *et al*., 1994) or as part of PAIs on the chromosome (Knapp *et al*., 1984, 1986; Falbo *et al*., 1993; Blum *et al*., 1995; Swenson *et al*., 1996). Similar findings have been reported for clostridial neurotoxin genes (Hauser *et al*., 1992; Whelan *et al*., 1994; Minton, 1995), for some enterotoxins, the exfoliative toxins A and B, as well as for ADP-ribosyltransferases B and C (EDIN-B, EDIN-C) of staphylococci (Altboum *et al*., 1985; Betley and Mekalanos, 1985; Couch *et al*., 1988; Johns and Khan, 1988; Zhang *et al*., 1998; Bayles and Iandolo, 1989; Iandolo, 1989; Yamaguchi *et al*., 2001; Yamaguchi *et al*., 2002; Alouf and Müller-Alouf, 2003). The cytolethal distending toxin (CDT) is produced by many pathogenic *E. coli* (Johnson *et al*., 2002; Tóth *et al*., 2003). Whereas the *cdt*-III determinant of septicemic *E. coli* isolate 1404 has been described to be plasmid-encoded (Peres *et al*., 1997), highly similar genes have been detected in the *E. coli* O157:H$^-$ strain 493/89 chromosome where they are flanked by genes with homology to late genes of lambdoid bacteriophages. This indicates that *cdt*-III may have been acquired by bacteriophage transduction (Janka *et al*., 2003).

The location of identical or highly related toxin encoding genes on different genetic elements raises the question of that whether toxin determinants may be part of transposons or other genetic structures that have the capacity to jump between different genetic elements. A transposon location was only reported for the ST enterotoxin structural genes of *E. coli*, which have been found on Tn*1681* (So and McCarthy, 1980) and on Tn*4521* (Lee *et al*., 1985; Hu *et al*., 1987; Hu and Lee, 1988). Interestingly, both enterotoxin genes (*sta* and *stb*), earlier described as part of different transposons, have been reported to be linked within a 40-kb mobile DNA region of virulence plasmid pTC isolated from a porcine enterotoxigenic *E. coli* strain (Fekete *et al*., 2003). Recently, the *Bacteroides fragilis* pathogenicity islet carrying the *B. fragilis* toxin (*bft*) gene has been described to be part of a new family of conjugative transposons. This may explain how this gene could be transferred from toxigenic to non-toxigenic *B. fragilis* isolates (Franco, 2004). Many protein toxin genes such as those encoding α-hemolysin, CNF1, LT enterotoxins, cholera toxin, and others are located next to intact IS elements, which may form complex transposons similar to Tn*5* or Tn*10*.

## TOXINS ENCODED BY PATHOGENICITY ISLANDS

### Pathogenicity islands

Virulence genes are frequently located on mobile or formerly mobile genetic elements, including PAIs (Hacker and Kaper, 2000; Hacker and Kaper, 2002). PAIs have evolved from former lysogenic bacteriophages and plasmids and are defined as large genomic regions present in pathogenic variants but less frequently present in closely related non-pathogenic bacteria. They carry one or more virulence-associated gene(s), have a G+C content differing from that of the rest of the chromosome, and are frequently associated with tRNA genes as well as flanked by repeat structures. PAIs are often unstable and contain mobility genes coding for integrases or transposases. Virulence genes located on PAIs can be divided into several groups according to their function: (i) adherence factors enable bacteria to attach to host surfaces; (ii) siderophores ensure sufficient solubilization and uptake of Fe (III) ions; (iii) capsules avoid phagocytosis and protect against other defense lines of the host immune system; (iv) the endotoxin (LPS) of Gram-negative organisms activates the host complement pathway and is a potent inducer of inflammation; (v) exotoxins destroy or affect eukaryotic cells; (vi) invasins mediate bacterial entry into eukaryotic cells; and (vii) type III and IV secretion systems are required for the targeted delivery of toxins or modulins into eukaryotic cells and modulate host contact, interfere with the host signal transduction pathways, promote apoptosis and entry into non-phagocytic cells. Similar structures have since been discovered in many Gram-negative and Gram-positive human, animal, and plant pathogens. However, several of these traits can be important for non-pathogenic, symbiotic, and environmental bacteria alike (Dobrindt *et al*., 2004).

### Pathogenicity island-encoded toxins

#### *Enterobacteria*

Initially, PAIs were discovered in uropathogenic *E. coli* (UPEC) by Goebel, Hacker, and co-workers (Hacker *et al*., 1990; Blum *et al*., 1994). Typical PAI-encoded protein toxins of UPEC and other extraintestinal pathogenic *E. coli* (ExPEC) are α-hemolysin and the cytotoxic necrotizing factor 1 (CNF-1) (see Table 3.4). In many ExPEC isolates, the corresponding *hly* and

**TABLE 3.4** Protein toxin-encoding pathogenicity islands (PAIs) of Gram-negative bacteria

| Organism | | Designation | Encoded toxin(s) | Other important encoded traits | Junction | Insertion site |
|---|---|---|---|---|---|---|
| *E. coli* 536 (UPEC) | O6:K15:H31 | PAI $I_{536}$ | α-hemolysin | put. adhesins | DR 16 bp | *selC* |
| *E. coli* 536 (UPEC) | O6:K15:H31 | PAI $II_{536}$ | α-hemolysin | P fimbriae (Prf), put. heat resistant agglutinin | DR 18 bp | *leuX* |
| *E. coli* 536 (UPEC) | O6:K15:H31 | PAI $III_{536}$ | hemoglobin protease (autotransporter serine protease) | S fimbriae (SfaI), salmochelin siderophore system | DR 46 bp | *thrW* |
| *E. coli* J96 (UPEC) | O4:$K^-$:H5 | PAI $I_{J96}$ | α-hemolysin | P fimbriae (Pap) | ? | *pheV* |
| *E. coli* J96 (UPEC) | O4:$K^-$:H5 | PAI $II_{J96}$ | α-hemolysin, cytotoxic necrotizing factor 1 (CNF1) | P fimbriae (Prs) | DR 135 bp | *pheU* |
| *E. coli* CFT073 (UPEC) | O6:K2:H1 | PAI $I_{CFT073}$ | α-hemolysin | P fimbriae (Pap) | DR 9 bp | *pheV* |
| *E. coli* C5 | O18:K1:H7 | PAI $I_{C5}$ | α-hemolysin, cytotoxic necrotizing factor 1 (CNF1), | P fimbriae (Prs), heat-resistant hemagglutinin | DR 18 bp | *leuX* |
| *E. coli* Ec222 | O78 | Vat-PAI | Vat cytotoxin (autotransporter serine protease) | | | *thrW* |
| *E. coli* 83/39 (REPEC) | O15:$H^-$ | LEE | Enterotoxin (Sen) | Type III secretion, invasion, put. adhesin | ND | *pheU* |
| *E. coli* E2348/69 (EPEC) | O127:H7 | EspC-PAI | EspC enterotoxin (autotransporter) | | ND | *ssrA* |
| *Shigella flexneri* | | SHI-1 (*she*) | Enterotoxin (Set), autotransporter serine protease (Pic) | | DR 22 bp (imperfect) | *pheV* |
| *Bacteroides fragilis* | | *B. fragilis* PAI | *B. fragilis* toxin (BFT) | - | ND | ND |
| *Neisseria gonorrhoeae* | | *atlA* locus | Cytotoxin | Serum resistance | ND | ND |
| *Bordetella pertussis* | | *ptx-ptl* locus | Pertussis toxin (Ptx) | | - | tRNA-Asp |
| *Photorhabdus luminescens* | | *tc* loci | Toxin complex (Tc) proteins | | ND | tRNA-Asp |
| | | *mcf* island | Makes caterpillars floppy (Mcf) protein | HecAB-like adhesin or hemolysin | ND | tRNA-Phe |
| | | *cnt* island | Cytonecrosis-like toxin (Cnt) | | ND | |
| | | *mt* island | Macrophage toxin (Mt) | | ND | |

DR- direct repeat
ND- not determined

*cnf*1 determinants are closely associated, thus underlining a possible co-evolution of these genes (Falbo *et al*., 1992; Swenson *et al*., 1996; Bingen-Bidois *et al*., 2002).

Genes coding for autotransporters are usually located on mobile genetic elements, such as plasmids, bacteriophages, and PAIs. Autotransporters constitute a distinct protein family that represent virulence factors, e.g., adhesions, proteases, and toxins (Guyer *et al*., 2000; Dutta *et al*., 2002; Henderson and Nataro, 2001). Vat, a Serine Protease Autotransporter of Enterobacteriaceae (SPATE), is a vacuolating toxin and was initially isolated from avian pathogenic *E. coli* strain Ec222. This gene is located on a 22-kb PAI, chromosomally inserted at the tRNA locus *thrW* that also serves as a chromosomal insertion site for several other

PAIs and bacteriophages. The Vat toxin induces cytotoxic effects as known for the VacA toxin of *Helicobacter pylori* (Parreira and Gyles, 2003). Vat shows high homology to other PAI-encoded autotransporters of pathogenic *E. coli*, e.g., the putative hemoglobin protease Hbp (accession numbers: Q83W66 and Q8FKM0) of uropathogenic *E. coli* O6 strains (95% identity on the protein level) (Dobrindt *et al*., 2002; Welch *et al*, 2002) or the hemagglutinin Tsh (accession number: Q47692) of an avian pathogenic *E. coli* isolate (78% identity on the protein level) (Stathopoulos *et al*., 1999). The enterotoxin EspC is also an autotransporter protein and is encoded by the *espC*-PAI in EPEC strains (Mellies *et al*., 2001).

Another protein toxin–encoding gene is located on a pathogenicity island of *Shigella flexneri*, termed *she* locus. The *she* locus of *S. flexneri* 2a codes for the protein ShMu, which has hemagglutinin and mucinase activities. ShMu shares homology with the virulence-related immunoglobulin A protease-like family of secreted proteins. Within the *she* locus, the two *Shigella* enterotoxin 1 encoding genes *set1B* and *set1A* are tandemly located in the opposite orientation. This element is part of a 51-kb deletable chromosomal element, which has been termed *she* PAI. The flanking regions of this PAI contain several IS elements like IS*2*, IS*600*, and a copy of IS*629*, which was disrupted by the insertion of a bacterial group II intron (Rajamukar *et al*., 1997). The *Shigella* enterotoxin gene (*senA*) has also been detected on a "locus of enterocyte effacement" (LEE)-PAI variant of a rabbit EPEC isolate (Tauschek *et al*., 2002).

### *Other Gram-negative bacteria*

*Photorhabdus luminescens* is pathogenic to a wide range of insects and has a complex symbiotic relationship with nematodes. The *P. luminescens* genome comprises more toxin genes than any other bacterial genome sequenced so far. Additionally, a large number of adhesins, proteases, and lipases, which also may be expressed during the pathogenic phase of its complex lifecycle, was predicted from the complete genome sequence. A multitude of so-called "toxin complex" (*tc*) loci exist on the chromosome of *P. luminescens* strains. All of them code for different high-molecular-weight insecticidal Tc toxins that exhibit oral and injectable activity against several insects. It is anticipated that some of the Tc proteins may function to destroy the insect midgut (ffrench-Constant *et al*., 2000). The so-called *tcd* locus inhibits several features of a pathogenicity island or similar (formerly) mobile genetic element. This DNA stretch comprises multiple tandem repeats of *tcdAB*-like genes interspersed with *tcc*-like sequences. The entire locus is chromosomally inserted at a tRNA-Asp gene and is also associated with multiple enteric repetitive intergenic consensus (ERIC)–like sequences and with a *tcdA*-like pseudogene. This indicates that the *tcdAB* island represents an unstable DNA region (ffrench-Constant *et al*., 2003; Duchaud *et al*., 2003). This is supported by the fact that deletions within this locus have been observed in other *Photorhabdus* strains (Waterfield *et al*., 2002). The genetic structure of "*tc*-island" sequences in several *Photorhabdus* strains is variable, and furthermore, *tc*-like genes are localized on the chromosome or a plasmid of pathogenic microorganisms, e.g., *Xenorhabdus* spp., *Serratia entomophila*, *Yersinia pestis, Ps. syringae pv. tomato, Fibrobacter succinogenes, Treponema denticola*, several of which are insect-associated. Two other toxin determinants are localized on PAI-like structures (see Table 3.4). The *mcf* gene coding for the probably pro-apoptotic Mcf protein is inserted near a tRNA-Phe gene and located next to the *palAB* genes that code for a HecAB-like adhesin or hemolysin. The chromosomal insertion site as well as the number of *mcf* loci differs between individual *Photorhabdus* strains. Cytonecrosis-like toxin (*cnt*-like) and macrophage toxin-like (*mt*-like) genes have also been described to be located on chromosomal islands (ffrench-Constant *et al*., 2000; ffrench-Constant *et al*., 2003; Duchaud *et al*., 2003).

*Bordetella pertussis*, the causative agent of whooping cough, secretes several toxins implicated in this disease. The major virulence factor produced by *B. pertussis* is the pertussis toxin PTX. The five genes encoding the different subunits of the toxin (*ptx*) and the nine accessory genes required for the PTX (type IV-) secretion system (*ptl*) are organized as a polycistronic operon (Locht and Keith, 1986; Weiss *et al*., 1993; Farizo *et al*., 1996), which has some characteristics of a PAI. In *B. pertussis*, the upstream region of the *ptx-ptl* locus was found to contain a truncated IS element similar to IS*481* of *B. pertussis*. The last gene of *ptx-ptl* locus (*ptlH*) is followed by a tRNA gene coding for an asparagine-specific tRNA and by an inverted repeat. When the chromosomal insertion point of the *ptx-ptl* locus of *B. pertussis* is compared to that of *B. parapertussis* and *B. bronchiseptica*, this site appears to be different in *B. pertussis* from *B. parapertussis* and *B. bronchiseptica* (Antoine *et al*., 1998).

Enterotoxigenic strains of *Bacteroides fragilis* produce a metalloprotease toxin designated fragilysin or *B. fragilis* toxin (BFT). The encoding gene is located on a DNA region termed a "fragilysin pathogenicity islet" (see below), which contains an open reading frame coding for a second metalloprotease (MPII) and another ORF that encodes a protein with homology to a snake cytotoxin. The fragilysin pathogenicity islet is 6,033 bp in size, contains nearly perfect 12-bp direct repeats near

both ends of the islet, and is not present in non-toxigenic strains of *Bacteroides fragilis* (Moncrief *et al*., 1998). A recent study demonstrated that the *B. fragilis* pathogenicity islet is contained in putative new conjugative transposons (CTn). If these CTns are transmissible, the *bft* gene could be transferred to non-toxigenic *B. fragilis* strains (Franco, 2004).

### *Gram-positive bacteria*

The majority of the toxins encoded by pathogenicity islands have been described for Gram-negative bacteria. In the last few years, however, an increasing number of Gram-positive bacteria with PAIs have been detected (see Table 3.5).

In *Staphylococcus aureus*, several types of island-like chromosomal regions have been characterized that can be distinguished due to their mobility (Lindsay and Holden, 2004). *S. aureus* pathogenicity islands (SaPIs) can move with high frequency, which is mediated by helper phages, whereas other island-like DNA stretches are extremely stable. Nine SaPIs have been identified in human and bovine isolates (Kuroda *et al*., 2001; Baba *et al*., 2002; Holden *et al*., 2004; Lindsay *et al*., 1998; Yarwood *et al*., 2002; Fitzgerald *et al*., 2001; Ubeda *et al*., 2003). These SaPIs often carry the toxic shock syndrome toxin 1 (TSST-1)–encoding gene (*tstH*). SaPI1, for example, is 15.2 kb in size and is flanked by 17 bp direct repeats. This island integrates site- and orientation-specific into the chromosome and also contains, in addition to *tstH*, a gene with homology to another superantigenic toxin (*ent*), a *D. nodosus vapE* homologue as well as a putative integrase gene (Lindsay *et al*., 1998). The staphylococcal enterotoxins SEB, SEC, SEK, SEL, and SEP are also SaPI-encoded, e.g., on SaPI3, SaPI4, SaPIbov (Alouf and Müller Alouf, 2003). Furthermore, the *S. aureus* exfoliative toxin D (ETD) is, together with the ADP-ribosyltransferase EDIN-B, encoded on a pathogenicity island (Yamaguchi *et al*., 2002). Additionally, there are other extremely stable island-like regions within the *S. aureus* chromosome that may carry determinants coding for superantigenic staphylococcal exotoxin-like proteins (SET), proteases, leukocidins, lantibiotics, lipoproteins, and proteases. They are always localized in the same chromosomal location. However, they can markedly differ with regard to gene content and structural organization between individual strains (Kuroda *et al*., 2001; Baba *et al*., 2002; Fitzgerald *et al*., 2003).

The cytolysin of *Enterococcus faecalis* contributes to the severity of enterococcal infection and is distantly related to lantibiotics (Shankar *et al*., 2004). The corresponding *cyl* genes can be located on either pheromone-responsive plasmids, e.g., pAD1 (Haas *et al*., 2002) or on the chromosome within a PAI. In *E. faecalis* strain MMH594, the cytolysin operon is part of a 150-kb PAI that also encodes other virulence-associated factors, e.g., the surface proteins Esp and aggregation substance (Ike and Clewell, 1992; Shankar *et al*., 2002). Extensive nucleotide sequence identity between the cytolysin operons of pAD1 and strain MMH594 suggest that the PAI-located gene cluster resulted from a transfer of the operon from pAD1 into the chromosome of strain MMH594 (Shankar *et al*., 2004). Gilmore and coworkers were able to show that a cytolysin- and Esp-positive *E. faecalis* strain can develop into a cytolysin- and Esp-negative variant at a high frequency, most likely due to homologous recombination between IS*905*-like elements located within the *cyl* operon and downstream of the *esp* gene (Shankar *et al*., 2002).

The pathogenic species of *Listeria* (*L. monocytogenes*, *L. ivanovii*), as well as the non-pathogenic *L. seeligeri*,

**TABLE 3.5** Protein toxin determinants located on pathogenicity islands of Gram-positive bacteria

| Organism | PAI designation | Encoded toxins | Other PAI-encoded genes | Boundary sequences |
|---|---|---|---|---|
| *S. aureus* | SaPI1 | Enterotoxin B, K, Q | *int*, orf11 (homology to *D. nodosus vapE*) | DR 17 bp |
| | SaPI2 | Toxic shock syndrome toxin 1, enterotoxin L, L2, C3, C4 | | |
| | SaPI3 | Enterotoxin C4, L2 | | |
| | SaPIbov | Toxic shock syndrome toxin-1, enterotoxin C | | DR 74 bp |
| | *etd*-PAI | Exfoliative toxin D | orf2 (homology to serine proteases), *edin-B* | DR 5 bp |
| *Enterococcus faecalis* | *E. faecalis* PAI | Cytolysin | Surface protein (Esp), aggregation substance | DR 10 bp |
| *L. monocytogenes* | LIPI-1 | Listeriolysin | PrfA-dependent virulence gene cluster (phospholipases, ActA) | - |
| | LIPI-2 | - | Internalins, sphingomyelinase C | - |
| *L. ivanovii* | Pathogenicity locus | Enterotoxin | | |
| *Cl. difficile* | | Cytotoxin | - | - |

DR- direct repeat

harbor a 10-kb virulence cluster containing the listeriolysin gene (*hly*), which is flanked on one side by the *plcA-prfA* region and on the other side by the lecithinase operon, which are required for intercellular spread during infection (Portnoy *et al*., 1992; Gouin *et al*., 1994). In *L. seeligeri*, this gene cluster is not properly expressed due to a reduced transcription of *prfA*, which encodes the transcriptional activator of *Listeria* virulence genes. The same genes that are located at both ends of the virulence cluster of *Listeria* (*ldh*, *prs*) are present in avirulent strains, but instead of the virulence gene cluster, housekeeping genes are located between *ldh* and *prs* (Glaser *et al*., 2001). Another putative pathogenicity region of *Listeria* has recently been described. The *inlC* gene of *L. monocytogenes* and the *i-inlC* gene of *L. ivanovii* show high sequence similarities, but they differ with respect to their chromosomal localization. Whereas *inlC* of *L. monocytogenes* is a monocistronic gene located between two housekeeping genes (*rplS* and *infC*), the *i-inlC* of *L. ivanovii* is associated with another small internalin gene (*i-inlD*) and has been inserted into a minor threonine-specific tRNA as part of a larger DNA fragment. This tRNA belongs to a rRNA/tRNA locus with a similar organization to the *rrnB* locus of *B. subtilis* (Engelbrecht *et al*., 1998).

The 19-kb *Clostridium difficile* pathogenicity locus (PaLoc), which exhibits several features of a PAI, is specific for virulent strains of *Cl. difficile* and comprises the genes encoding the enterotoxin (*tcdA*), the cytotoxin (*tcdB*), as well as the accessory genes *tcdC–E* (Braun *et al*., 1996). The integration site of the PaLoc was defined as a 115-bp fragment, which forms a 20-bp hairpin loop. This stretch is only found in non-toxinogenic strains and is replaced by the PaLoc in toxinogenic strains (Braun *et al*., 1996).

## Instability of pathogenicity islands

The flanking regions of several PAIs are often characterized by the occurrence of direct repeats what can be involved in recombinational events leading to the deletion of the PAI (see Table 3.4, 3.5). In case of the PAIs of UPEC strains 536 and one PAI of strain J96, respectively, it has been shown that they can spontaneously delete from the chromosome at high frequencies upon site-specific recombination between short direct repeats (Blum *et al*., 1994; Blum *et al*., 1995; Swenson *et al*., 1996; Middendorf *et al*., 2004). IS elements or RS-sequences can also be responsible for the excision and maybe integration of genetic elements. In *Y. pestis*, homologous recombination between IS*100* elements flanking the HPI leads to the deletion of this PAI (Fetherston *et al*., 1992). PAI excision is accompanied by the loss of PAI-encoded determinants (*cnf*1, *hly*, *pap*, *pgm* locus) and reduced virulence of the resulting strains (Fetherston *et al*., 1992; Blum *et al*., 1994; Blum *et al*., 1995; Swenson *et al*., 1996). It is suggested that such deletion events may represent a specific form of adaptation of these pathogens to certain niches, hosts, tissues or during transition of the pathogen from an acute state of infection to a chronic state where expression of highly antigenic factors may be disadvantageous. More generally, pathogens with a relatively high genetic flexibility may be more competent in the colonization of new ecological niches and may have a selective advantage over organisms with less flexible genomes (Hacker *et al*., 2003).

Genome plasticity and gene loss, e.g., in *E. coli*, can be detected in different nuances: in addition to the loss of complete PAIs, deletion of distinct regions within genomic islands may frequently occur due to homologous recombination of IS elements and also results in phenotypic changes. Comparison of the genome content of non-pathogenic and uropathogenic *E. coli* O6 strains demonstrated that the gene content and the general genetic structure of the *pheV*-associated islands of UPEC strain CFT073 and non-pathogenic strain Nissle 1917 are highly similar. In the latter strain, however, the α-hemolysin and P fimbriae determinants, present on this island in UPEC strain CFT073, are deleted. This is supposed to be one of the reasons for this strain's non-pathogenic phenotype relative to strain CFT073 (Grozdanov *et al*., 2004).

# CONCLUSION

## Evolution of new pathogenic variants caused by pathogenicity islands and mobile genetic elements

Genes encoding toxins and other virulence factors can be located on PAIs and/or on mobilizable genetic elements such as plasmids, transposons, and bacteriophages. This is especially true for microorganisms, which lack the capacity to take up foreign DNA because of natural competence. The presence of so-called "mobility sequences," e.g., direct repeats, sequences with homology to integrases or to plasmid origins of replication near the borders of PAIs, imply that PAIs are derived from integrated mobile elements such as bacteriophages and plasmids (Hacker and Kaper, 2002). Plasmids and phages are able to form co-integrates. This has been found frequently in *Streptomyces* (Leblond and Decaris, 1994) and may be an explanation for the existence of features of plasmids and phages on PAIs. Therefore, integrated plasmids and bacteriophages have been considered as "PAI precursors" or "Pre-PAIs." This underlines the strong

mutual dependence between plasmids, bacteriophages, and pathogenicity islands. This interdependence not only contributes to the genetic flexibility of bacteria carrying these genetic elements but to the fast evolution of new pathogenic variants as well (see also Figure 3.1) (Groisman and Ochman, 1996; Hacker *et al*., 2003; Dobrindt *et al*., 2004).

The spread of specific genomic regions through lateral gene transfer followed by integration into the bacterial genome is a mechanism by which new variants of microbes could be generated. This can occur by lysogenic conversion in which the prophage confers specific changes in the bacterial phenotype or by the uptake of plasmids or DNA fragments by conjugation or transformation. A genomic island or its more specified variant (PAI) may be the result of such transfer processes and subsequently generated point mutations. Such spontaneous point mutations in "mobility genes" may lead to a fixation of laterally acquired genes in specific strains. Under specific selective pressure a "homing" of new variants by inactivation of mobility genes could be advantageous, resulting in some structures of PAIs described in this article (see Figure 3.1). An interesting exception is *Cl. perfringens*: the complete genome sequence revealed that all known and putative virulence-associated genes of this pathogen are not located on PAIs, as IS element-, transposon- or bacteriophage-related sequences have not been found in their flanking regions. In addition, their G+C content does not significantly differ from that of other genes, indicating that acquisition of these genes via horizontal gene transfer did not occur or was not a recent event (Shimizu *et al*., 2002). Another exception is the superantigen-encoding genes *ypm* (*Y. pseudotuberculosis*-derived mitogen) of *Y. pseudotuberculosis* that, unlike other superantigenic toxin genes, are not associated with mobile genetic elements but are localized in unstable regions of the chromosome. This explains why deletion of the *ypm* genes can occur with significantly increased frequency (Carnoy *et al*., 2002).

## Horizontal gene transfer and the evolution of toxin families

The existence of toxin families with common properties and sequence homologies in the relevant genes argues that such a distribution of genetic information is a result of lateral gene transfer. The most prominent examples of genetically related toxin families are ADP ribosylating toxins (diphtheria toxin, pertussis toxin, cholera toxin, *E. coli* LT, *Pseudomonas* exotoxin A and S, and others) and other AB toxins (Shiga toxins), pore-forming RTX toxins (i.e., various hemolysins, *Pasteurella haemolytica* leucotoxin, *B. pertussis* adenylate cyclase-hemolysin), clostridial neurotoxins (*Cl. botulinum* and *Cl. tetani* neurotoxins), enterobacterial autotransporters and proteins secreted by a type III secretion system. Many of them are encoded on plasmids, phages, or pathogenicity islands. There are various reasons that demonstrate that members of these toxin families can be transferred by mobile genetic elements or may have formerly been transferred by them. Other examples, such as the occurrence of many different proteins that share functional features of superantigens, may argue for convergent evolution of proteins with similar toxic characteristics in different bacteria. This is less probable in the case of toxin families than the acquisition by horizontal gene transfer because of the complexity of mutational and evolutionary events on the DNA level that would have to occur in order to result in functionally and structurally similar proteins. In *S. aureus*, the superantigenic toxins SEG and SEI-encoding genes (*seg*, *sei*) are linked by a DNA region, the enterotoxin gene cluster (*egc*), that comprises three enterotoxin-like open reading frames (ORFs) *sek*, *sel*, and *sem*, as well as two pseudogenes. The *egc* gene cluster was detectable in many other *S. aureus* isolates, and phylogenetic analyses of staphylococcal enterotoxin-encoding genes implied that they all potentially derive from the *egc* cluster. It has been speculated that the existing great variety of superantigens in *S. aureus* may represent an enterotoxin "nursery" that enables expression of a broad variety of toxin variants when needed (Jarraud *et al*., 2001). Phylogenetic analysis of staphylococcal exotoxin-like protein determinants (*set*) localized on island-like region RD13 within a variety of *S. aureus* isolates that gene loss as well as recombination contributed to the diversification of this island (Fitzgerald *et al*., 2003).

The great similarity in the protein sequences of the *E. coli* LT and *V. cholerae* CT led to the assumption that CT, LT I, and LT II originally derived from the same ancestral gene. Other toxins immunologically related to CT can be found in *Salmonella*, *Pseudomonas*, *Campylobacter*, and *Aeromonas*. This may suggest that this is the result of different evolutionary pathways that this gene followed in different species (Rappuoli and Pizza, 1991).

The pattern of distribution of genes involved in toxin expression on plasmids relative to the chromosome indicates that they may derive from a common ancestor, such as the type III protein secretion systems of various bacterial animal or plant pathogens (*Yersinia spp*., *Shigella flexneri*, *Salmonella typhimurium*, EPEC, *Ps. aeruginosa*, *Chlamydia spp*., *Erwinia spp*., *Ps. syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris*) (Mecsas and Strauss, 1996; Yahr *et al*., 1996; Hsia *et al*., 1997; Bonas, 1994; Collmer and Bauer, 1994). Although

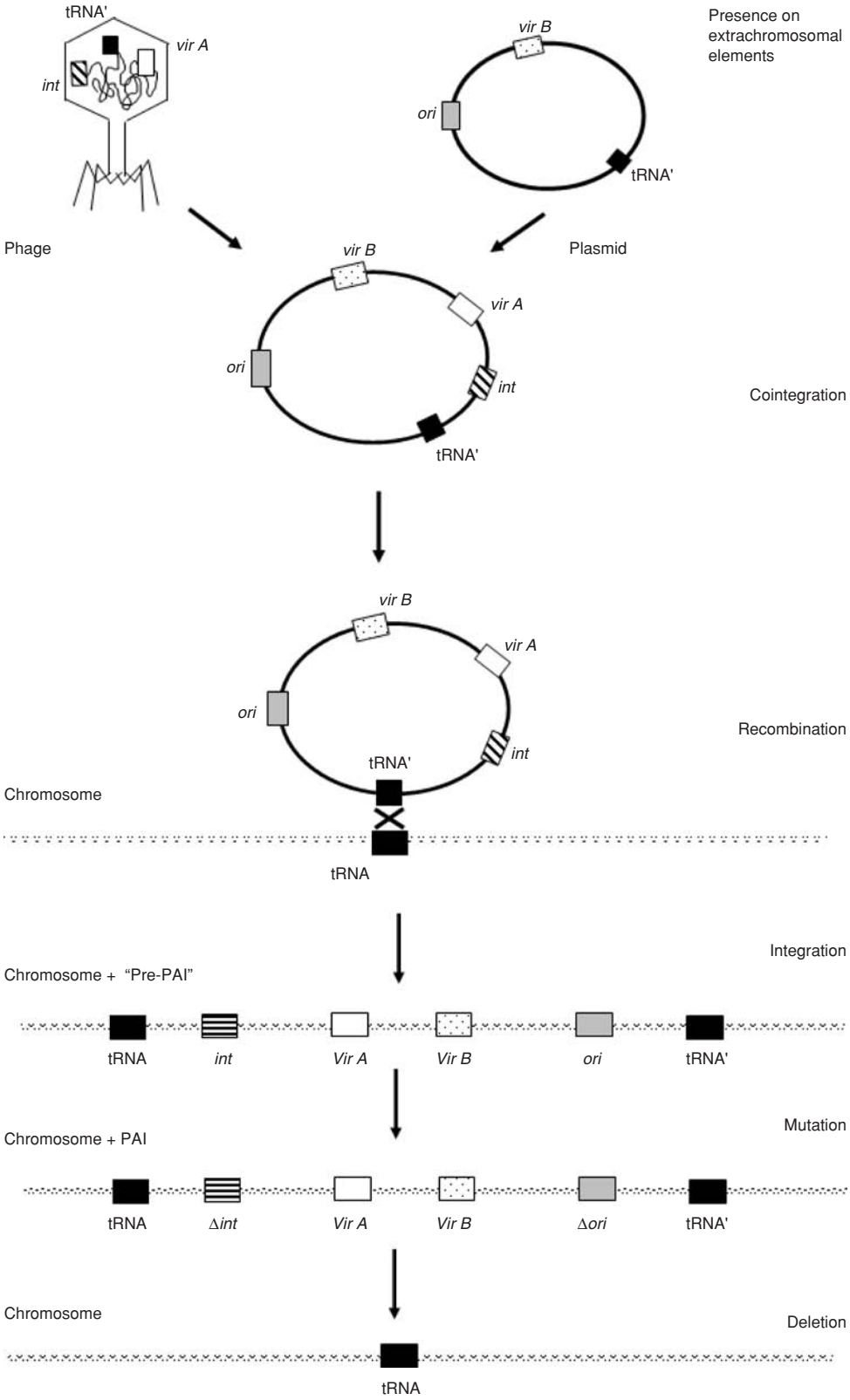



**FIGURE 3.1** Model illustrating the impact of mobile genetic elements on the evolution of pathogenicity islands (PAIs). A chromosomal integration of mobile genetic elements into the chromosome can occur upon recombination of homologous sites. A stabilization of the integrated DNA may occur by mutations in integrase genes (*int*) and/or origins of replication (*ori*). The deletion of PAIs may lead to avirulent variants.

the pathogenicity mechanisms and therefore the secreted effector molecules of these bacteria differ, the type III secretion apparatus is conserved and effector molecules from one pathogen can be translocated by another system when the appropriate chaperons are present (Mecsas and Strauss, 1996).

Taken together, virulence determinants in general and protein toxin genes in particular are frequently encoded by mobile genetic elements. They are encoded on large genomic regions as well, designated pathogenicity islands (PAIs), which often contain clusters of virulence-associated genes. These genetic elements with the capacity to spread by horizontal gene transfer contribute to the rapid evolution of bacterial pathogens as the rearrangement, excision, and acquisition of large genomic regions creates new pathogenic variants. The occurrence of protein toxin–encoding genes on various interdepending mobile genetic elements, their ability to delete from and integrate into chromosomal DNA, and the existence of toxin families among a wide variety of bacterial species show that the continuing process of the evolution of new pathogens is significantly connected with the transfer of foreign DNA harboring toxin determinants.

## ACKNOWLEDGMENTS

Our own work related to the subject of this article is supported by the DFG (Sonderforschungsbereich 479), the Bayerische Forschungsstiftung, and the Fonds der Chemischen Industrie.

## REFERENCES

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological, and clinical aspects. *Int. J. Med. Microbiol*. **292,** 429–440.

Altboum, Z., Hertman, I. and Sadrid, S. (1985). Penicillinase plasmid-linked genetic determinants for enterotoxin B and $C_1$ production in *Staphylococcus aureus*. *Infect. Immun*. **47,** 514–521.

Antoine, R., Raze, D. and Locht, C. (1998). The pertussis toxin locus has some features of a pathogenicity island. *Zentralbl. Bakteriol*. [Suppl.] **29,** 393–394.

Artiushin, S.C., Timoney, J.F., Sheoran, A.S. and Muthupalani, S.K. (2002). Characterization and immunogenicity of pyrogenic mitogens SePE-H and SePE-I of *Streptococcus equi*. *Microb. Pathog*. **32,** 71–85.

Asakura, H., Makino, S., Kobori, H., Watarai, M., Shirahata, T., Ikeda, T. and Takeshi, K. (2001). Phylogenetic diversity and similarity of active sites of Shiga toxin (stx) in Shiga toxin-producing *Escherichia coli* (STEC) isolates from humans and animals. *Epidemiol. Infect*. **127,** 27–36.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K. and Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359,** 1819–1827.

Banks, D.J., Porcella, S.F., Barbian, K.D., Beres, S.B., Philips, L.E., Voyich, J.M., DeLeo, F.R., Martin, J.M., Somerville, G.A. and Musser, J.M. (2004). Progress toward characterization of the group A *Streptococcus* metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain. *J. Infect. Dis*. **190,** 727–738.

Barksdale, W.L. and Pappenheimer, A.M. Jr. (1954), Phage-host relationships in nontoxinogenic and toxinogenic diphtheria bacilli. *J. Bacteriol*. **67,** 220–232.

Bauer, M.E. and Welch, R.A. (1996). Characterization of an RTX-toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun*. **64,** 167–175.

Bayles, K.W. and Iandolo, J.J. (1989). Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin *D. J. Bacteriol*. **171,** 4799–4806.

Bentley, S.D. and Parkhill, J. (2004). Comparative genomic structure of prokaryotes. *Annu. Rev. Genet*. **38,** 771–791.

Beres, S.B., Sylva, G.L., Barbian, K.D., Lei, B., Hoff, J.S., Mammarella, N.D., Liu, M.Y., Smoot, J.C., Porcella, S.F., Parkins, L.D., Campbell, D.S., Smith, T.M., McCormick, J.K., Leung, D.Y., Schlievert, P.M. and Musser, J.M. (2002). Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc. Natl. Acad. Sci. USA* **99,** 10078–10083.

Berg, D.E. (1989). Mobile DNA. *ASM Press*, Washington, D.C.

Betley, M.J. and Mekalanos, J.J. (1985). Staphylococcal enterotoxin A is encoded by phage. *Science* **229,** 185–187.

Beuscher, H.U., Rödel, F., Forsberg, Å. and Röllinghoff, M. (1995). Bacterial evasion of host immune response: *Yersinia enterocolitica* encodes a suppressor for tumor necrosis factor alpha expression. *Infect. Immun*. **63,** 1270–1277.

Bingen-Bidois, M., Clermont, O., Bonacorsi, S., Terki, M., Brahimi, N., Loukil, C., Barraud, D. and Bingen, E. (2002). Phylogenetic analysis and prevalence of urosepsis strains of *Escherichia coli* bearing pathogenicity island-like domains. *Infect. Immun*. **70,** 3216–3226.

Bliska, J.B., Clemens, J.C., Dixon, J.E. and Falkow, S. (1992). The *Yersinia* tyrosine phosphatase: specificity of a bacterial virulence determinant for phosphoproteins in the J774A.1 macrophage. *J. Exp. Med*. **176,** 1625–1630.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschäpe, H. and Hacker, J. (1994). Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infect. Immun*. **62,** 606–614.

Blum, G., Falbo, V., Caprioli, A. and Hacker, J. (1995). Gene clusters encoding the cytotoxic necrotizing factor 1, Prs-fimbriae and α-hemolysin form the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. *FEMS Microbiol. Lett*. **126,** 189–196.

Bonas, U. (1994). *hrp* genes of phytopathogenic bacteria. *Curr. Top. Microbiol. Immunol*. **192,** 79–98.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. and von Eichel Streiber, C. (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* **181,** 29–38.

Brüggemann, H., Bäumer, S., Fricke, W.F., Wiezer, A., Liesegang, H., Decker, I., Herzberg, C., Martinez-Arias, R., Merkl, R., Henne, A. and Gottschalk, G. (2003). The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc. Natl. Acad. Sci. USA* **100,** 1316–1321.

Brüssow, H., Canchaya, C. and Hardt, W.D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol. Mol. Biol. Rev*. **68,** 560–602.

Buchrieser, C., Glaser, P., Rusniok, C., Nedjari, H., D'Hauteville, H., Kunst, F., Sansonetti, P. and Parsot, C. (2000). The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol. Microbiol*. **38,** 760–771.

Carnoy, C., Floquet, S., Marceau, M., Sebbane, F., Haentjens-Herwegh, S., Devalckenaere, A. and Simonet, M. (2002). The superantigen gene *ypm* is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis*. *J. Bacteriol*. **184,** 4489–4499.

Collier, R.J. (1982). Structure and activity of diphtheria toxin. In: ADP-ribosylation reactions, (eds.) D. Hayashi and K. Ueda, Academic Press, New York, pp. 575–592.

Collmer, A. and Bauer, D.W. (1994). *Erwinia chrysanthemi* and *Pseudomonas syringae*: plant pathogens trafficking in extracellular virulence proteins. *Curr. Top. Microbiol. Immunol*. **192,** 43–78.

Colonna, B., Casalino, M., Fradiani, P.A., Zagaglia, C., Naitza, S., Leoni, L., Prosseda, G., Coppo, A., Gherlardini, P. and Nicoletti, M. (1995). H-NS regulation of virulence gene expression in enteroinvasive *Escherichia coli* harboring the virulence plasmid integrated into the host chromosome. *J. Bacteriol*. **177,** 4703–4712.

Couch, J.L., Soltis, M.T. and Betley, M.J. (1988). Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. *J. Bacteriol*. **170,** 2954–2960.

De la Cruz, F., Müller, D., Ortiz, J.M. and Goebel, W. (1980). Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. *J. Bacteriol*. **143,** 825–833.

Deonier, R.C. (1996). Native insertion sequence elements: locations, distributions, and sequence relationships. In: Escherichia coli *and* Salmonella typhimurium. *Cellular and Molecular Biology*. (eds F.C. Neidhardt, R. Curtis III, J.L. Ingraham, E.C.C. Lin, K. Brooks Low, Jr., B. Magasanik, W.S. Reznikoff, M Riley, M Schaechter, H.E. Umbarger), pp.2000–2011, ASM Press, Washington D.C.

Dobrindt, U., Blum-Oehler, G., Nagy, G., Schneider, G., Johann, A., Gottschalk, G. and Hacker, J. (2002). Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic *Escherichia coli* strain 536. *Infect. Immun*. **70,** 6365–6372.

Dobrindt, U., Hochhut, B., Hentschel, U. and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol*. **2,** 414–424.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin, A. and Kunst, F. (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol*. **21,** 1307–1313.

Dubreuil, J.D. (1997). *Escherichia coli* STb enterotoxin. *Microbiology* **143,** 1783–1795.

Echeverria, P., Seriwatana, J., Taylor, D.N., Tirapat, C. and Rowe, B. (1985). *Escherichia coli* contains plasmids coding for heat-stable b, other enterotoxins, and antibiotoc resistance. *Infect. Immun*. **48,** 843–846.

Eklund, M.W., Poysky, F.T., Reed, S.M. and Smith, C.A. (1971). Bacteriophages and toxigenicity of *Clostridium botulinum* type D. *Nature (London) New Biol*. **235,** 16–18.

Eklund, M.W., Poysky, F.T., Mseitif, L.M. and Strom, M.S. (1988). Evidence for plasmid-mediated toxin production and bacteriocin production in *Clostridium botulinum* type G. *Appl. Environ. Microbiol*. **54,** 1405–1408.

Engelbrecht, F., Dickneite, C., Lampidis, R., Götz, M., DasGupta, U. and Goebel, W. (1998). Sequence comparison of the chromosomal regions encompassing the internalin C genes (*inlC*) of *Listeria monocytogenes* and *L. ivanovii*. *Mol. Gen. Genet*. **257,** 186–197.

Falbo, V., Famiglietti, M. and Caprioli, A. (1992). Gene block encoding production of cytotoxic necrotizing factor 1 and hemolysin in *Escherichia coli* isolates from extraintestinal infections. *Infect. Immun*. **60,** 2182–2187.

Falbo, V., Pace, T., Picci, L., Pizzi, E. and Caprioli, A. (1993). Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. *Infect. Immun*. **61,** 4904–4914.

Farizo, K.M., Cafarella, T.G. and Burns, D.L. (1996). Evidence for a ninth gene, *ptlI*, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *J. Biol. Chem*. **271,** 31643–31649.

Faruque, S.M., Zhu, J., Asadulghani, Kamruzzaman, M. and Mekalanos, J.J. (2003). Examination of diverse toxin-coregulated pilus-positive *Vibrio cholerae* strains fails to demonstrate evidence for *Vibrio* pathogenicity island phage. *Infect. Immun*. **71,** 2993–2999.

Fekete, P.Z., Schneider, G., Olasz, F., Blum-Oehler, G., Hacker, J.H. and Nagy, B. (2003). Detection of a plasmid-encoded pathogenicity island in F18+ enterotoxigenic and verotoxigenic *Escherichia coli* from weaned pigs. *Int. J. Med. Microbiol*. **293,** 287–298.

Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A. and McLaughlin, R. (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. USA* **98,** 4658–4663.

Fetherston, J.D., Schuetze, P. and Perry, R.D. (1992). Loss of pigmentation phenotype in *Yersinia pestis* is due to the spontaenous deletion of 102 kb of chromosomal DNA, which is flanked by a repetitive element. *Mol. Microbiol*. **6,** 2693–2704.

Finn, C.W., Jr, Silver, R.P., Habig, W.H. and Hardegree, M.C. (1984). The structural gene for tetanus neurotoxin is on a plasmid. *Science* **224,** 881–884.

Fitzgerald, J.R., Monday, S.R., Foster, T.J., Bohach, G.A., Hartigan, P.J., Meaney, W.J. and Smyth, C.J. (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol*. **183,** 63–70.

Fitzgerald, J.R., Reid, S.D., Ruotsalainen, E., Tripp, T.J., Liu, M., Cole, R., Kuusela, P., Schlievert, P.M., Jarvinen, A. and Musser, J.M. (2003). Genome diversification in *Staphylococcus aureus*: Molecular evolution of a highly variable chromosomal region encoding the Staphylococcal exotoxin-like family of proteins. *Infect. Immun*. **71,** 2827–2838.

Franco, A.A. (2004). The *Bacteroides fragilis* pathogenicity island is contained in a putative novel conjugative transposon. *J. Bacteriol*. **186,** 6077–6092.

ffrench-Constant, R.H., Waterfield, N., Burland, V., Perna, N.T., Daborn, P.J., Bowen, D. and Blattner, F.R. (2000). A genomic sample sequence of the entomopathogenic bacterium *Photorhabdus luminescens* W14: potential implications for virulence. *Appl. Environ. Microbiol*. **66,** 3310–3329.

ffrench-Constant, R., Waterfield, N., Daborn, P., Joyce, S., Bennett, H., Au, C., Dowling, A., Boundy, S., Reynolds, S. and Clarke, D. (2003). *Photorhabdus*: towards a functional genomic analysis of a symbiont and pathogen. *FEMS Microbiol. Rev*. **26,** 433–456.

Friedrich, A.W., Bielaszewska, M., Zhang, W.L., Pulz, M., Kuczius, T., Ammon, A. and Karch, H. (2002). *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J. Infect. Dis*. **185,** 74–84.

Galyov, E.E., Håkansson, S., Forsberg, Å. and Wolf-Watz, H. (1993). A secreted protein kinase of *Yersinia pseudotuberculosis* is an indispensable virulence determinant. *Nature* **361,** 730–731.

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R. and Clewell, D.B. (1994). Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J. Bacteriol*. **176,** 7335–7344.

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couve, E., de Daruvar, A., Dehoux, P., Domann, E., Dominguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., Garcia-del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gomez-Lopez, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueno, E., Maitournam, A., Vicente, J.M., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., de Pablos, B., Perez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vazquez-Boland, J.A., Voss, H., Wehland, J. and Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science* **294,** 849–852.

Gouin, E., Mengaud, J. and Cossart, P. (1994). The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a non-pathogenic species. *Infect. Immun*. **62,** 3550–3553.

Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B. and Ivins, B.E. (1985). Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun*. **49,** 291–297.

Groisman, E.A. and Ochman, H. (1996). Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* **87,** 791–794.

Grozdanov, L., Raasch, C., Schulze, J., Sonnenborn, U., Gottschalk, G., Hacker, J. and Dobrindt, U. (2004). Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. *J. Bacteriol*. **186,** 5432–5441.

Dutta, P.R., Cappello, R., Navarro-Garcia, F. and Nataro, J.P. (2002). Functional comparison of serine protease autotransporters of enterobacteriaceae. *Infect. Immun*. **70,** 7105–7113.

Guyer, D.M., Henderson, I.R., Nataro, J.P. and Mobley, H.L. (2000). Identification of sat, an autotransporter toxin produced by uropathogenic *Escherichia coli*. *Mol. Microbiol*. **38,** 53–66.

Gyles, C.M. (1992). *Escherichia coli* cytotoxins and enterotoxins. *Can. J. Microbiol*. **38,** 734–746.

Gyles, C.M., So, M. and Falkow, S. (1974). The enterotoxin plasmids of *Escherichia coli*. *J. Infect. Dis*. **130,** 40–48.

Haas, W., Shepard, B.D. and Gilmore, M.S. (2002). Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. *Nature* **415,** 84–87.

Hacker, J. and Hughes, C. (1985). Genetics of *Escherichia coli* hemolysin. *Curr. Top. Microbiol. Immunol*. **118,** 139–162.

Hacker, J., Bender, L., Ott, M., Wingender, J., Lund, B., Marre, R. and Goebel, W. (1990). Deletions of chromosomal regions coding for fimbriae and hemolysins occur *in vivo* and *in vitro* in various extraintestinal *Escherichia coli* isolates. *Microb. Pathog*. **8,** 213–225.

Hacker, J. and Kaper, J.B. (2000). Pathogenicity islands and the evolution of microbes. *Annu. Rev. Microbiol*. **54,** 641–679.

Hacker, J. and Carniel, E. (2001). Ecological fitness, genomic islands, and bacterial pathogenicity. A Darwinian view of the evolution of microbes. *EMBO Rep*. **2,** 376–381.

Hacker, J. and Kaper, J. B. (2002). Pathogenicity islands and the evolution of pathogenic microbes. Springer: Berlin, Heidelberg, New York.

Hacker, J., Hentschel, U. and Dobrindt, U. (2003). Prokaryotic chromosomes and disease. *Science* **301,** 790–793.

Hale, T.L. (1991). Genetic basis of virulence in *Shigella* species. *Microbiol. Rev*. **55,** 206–224.

Hardy, K. (1986). *Bacterial Plasmids* (2nd edition). ASM Press, Washington, D.C.

Harnett, N.M. and Gyles, C.L. (1985). Linkage of genes for heat-stable enterotoxin, drug resistance, K99 antigen, and colicin in bovine and porcine strains of enterotoxigenic *Escherichia coli*. *Am. J. Vet. Res*. **46,** 428–433.

Hauser, D., Gibert, M., Boquet, P. and Popoff, M.R. (1992). Plasmid localization of a type E botulinal neurotoxin gene homologue in toxigenic *Clostridium butyricum* strains, and absence of this gene in non-toxigenic *C. butyricum* strains. *FEMS Microbiol. Lett*. **99,** 251–256.

Hayashi, T., Matsumoto, H., Ohnishi, M. and Terawaki, Y. (1993). Molecular analysis of a cytotoxin-converting phage, ϕCTX, of *Pseudomonas aeruginosa*: structure of the *attP-cos-ctx*-region and integration into the serine tRNA gene. *Mol. Microbiol*. **7,** 657–667.

Henderson, I.R. and Nataro, J.P. (2001). Virulence functions of autotransporter proteins. *Infect. Immun*. **69,** 1231–1243.

Hentschel, U. and Hacker, J. (2001). Pathogenicity islands: the tip of the iceberg. *Microbes Infect*. **3,** 545–548.

Herold, S., Karch, H. and Schmidt, H. (2004). Shiga toxin-encoding bacteriophages—genomes in motion. *Int. J. Med. Microbiol*. **294,** 115–121.

Hess, J., Wels, W., Vogel, M. and Goebel, W. (1986). Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. *FEMS Microbiol. Lett*. **34**, 1–11.

Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., Barron, A., Bason, N., Bentley, S.D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K.D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall, K., Ormond, D., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrell, B.G., Spratt, B.G. and Parkhill, J. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc. Natl. Acad. Sci. USA* **101**, 9786–9791.

Hsia, R.-C., Pannekoek, Y., Ingerowski, E. and Bavoil, P.M. (1997). Type III secretion genes identify a putative virulence locus of *Chlamydia*. *Mol. Microbiol*. **25**, 351–359.

Hu, S.T. and Lee, C.H. (1988). Characterization of the transposon carrying the STII gene of enterotoxigenic *Escherichia coli*. *Mol. Gen. Genet*. **214**, 490–495.

Hu, S.T., Yang, M.K., Spandau, D.F. and Lee, C.H. (1987). Characterization of the terminal sequences flanking the transposon that carries the *Escherichia coli* enterotoxin STII gene. *Gene* **55**, 157–167.

Iandolo, J.J. (1989). Genetic analysis of extracellular toxins of *Staphylococcus aureus*. *Annu. Rev. Microbiol*. **43**, 375–402.

Ike, Y. and Clewell, D.B. (1992). Evidence that the hemolysin bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. *J. Bacteriol*. **174**, 8172–8177.

Inoue, K. and Iida, H. (1970). Conversion to toxigenicity in *Clostridium botulinum* type C. *Jap. J. Microbiol*. **14**, 87–89.

Jackson, M.P. and Iandolo, J.J. (1985). Cloning and expression of the exfoliative toxin B gene from *Staphylococcus aureus*. *J. Bacteriol*. **166**, 574–580.

Janka, A., Bielaszewska, M., Dobrindt, U., Greune, L., Schmidt, M.A. and Karch, H. (2003). Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. *Infect. Immun*. **71**, 3634–3638.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M. and Lina, G. (2001). *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol*. **166**, 669–677.

Jett, B.D., Huycke, M.M. and Gilmore, M.S. (1994). Virulence of enterococci. *Clin. Microbiol. Rev.* **7**, 462–478.

Johns, B.M. and Khan, S.A. (1988). Staphylococcal enterotoxin B gene is associated with a discrete genetic element. *J. Bacteriol.* **170**, 4033–4039.

Johnson, J.R., Oswald, E., O'Bryan, T.T., Kuskowski, M.A. and Spanjaard, L. (2002). Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. *J. Infect. Dis.* **185**, 774–784.

Kaneko, J., Kimura, T., Narita, S., Tomita, T. and Kamio, Y. (1998). Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. *Gene* **215**, 57–67.

Kaspar, R.L. and Robertson, D.L. (1987). Purification and physical analysis of *Bacillus anthracis* plasmids pX01 and pX02. *Biochem. Biophys. Res. Commun.* **149**, 362–368.

Knapp, S., Hacker, J., Jarchau, T. and Goebel, W. (1986). Large, unstable inserts in the chromosome affect virulence properties of uropathogenic *Escherichia coli* O6 strain 536. *J. Bacteriol.* **168**, 22–30.

Knapp, S., Hacker, J., Then, I., Müller, D. and Goebel, W. (1984). Multiple copies of hemolysin genes and associated sequences in the chromosomes of uropathogenic *Escherichia coli* strains. *J. Bacteriol.* **159**, 1027–1033.

Knapp, S., Then, I., Wels, W., Michel, G., Tschäpe, H., Hacker, J. and Goebel, W. (1985). Analysis of the flanking regions from different hemolysin determinants of *Escherichia coli*. *Mol. Gen. Genet.* **200**, 385–392.

Kovach, M.E., Shaffer, M.D. and Peterson, K.M. (1996). A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. *Microbiology* **142**, 2165–2174.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240.

Laird, W.J., Aaronson, W., Silver, R.P., Habig, W.H. and Hardegree, M.C. (1980). Plasmid-associated toxigenicity in *Clostridium tetani*. *J. Infect. Dis.* **142**, 623.

Leblond, P. and Decaris, B. (1994). New insights into the genetic instability of *Streptomyces*. *FEMS Microbiol. Lett.* **123**, 225–232.

Lee, C.H., Hu, S.T., Swiatek, P.J., Moseley, S.L., Allen, S.D. and So, M. (1985). Isolation of a novel transposon which carries the *Escherichia coli* enterotoxin STII gene. *J. Bacteriol.* **162**, 615–620.

Levine, M.M. (1987). *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* **155**, 377–389.

Lindler, L.E., Plano, G.V., Burland, V., Mayhew, G.F. and Blattner, F.R. (1998). Complete DNA sequence and detailed analysis of the *Yersinia pestis* KIM5 plasmid encoding murine toxin and capsular antigen. *Infect. Immun.* **66**, 5731–5742.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N. and Novick, R.P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Mol. Microbiol.* **29**, 527–543.

Lindsay, J.A. and Holden, M.T. (2004). *Staphylococcus aureus*: superbug, super genome? *Trends Microbiol.* **12**, 378–385.

Locht, C. and Keith, J.M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232**, 1258–1264.

Lüneberg, E., Mayer, B., Daryab, N., Kooistra, O., Zähringer, U., Rohde, M., Swanson, J. and Frosch, M. (2001). Chromosomal insertion and excision of a 30 kb unstable genetic element is responsible for phase variation of lipopolysaccharide and other virulence determinants in *Legionella pneumophila*. *Mol. Microbiol.* **39**, 1259– 1271.

McShan, W.M., Tang, Y.-F. and Ferretti, J.J. (1997). Bacteriophage T12 of *Streptococcus pyogenes* integrates into the gene encoding a serine tRNA. *Mol. Microbiol.* **23**, 719–728.

Mainil, J.G., Jacquemin, E., Herault, F. and Oswald, E. (1997). Presence of *pap*-, *sfa*-, and *afa*-related sequences in necrotoxigenic *Escherichia coli* isolates from cattle: evidence for new variants of the AFA family. *Can. J. Vet. Res.* **61**, 193–199.

Marques, L.R.M., Peiris, J.S.M., Cryz, S.J. and O'Brien, A.D. (1987). *Escherichia coli* strains isolated from pigs with edema disease produce a variant of Shiga-like toxin II. *FEMS Microbiol Lett.* **44**, 33–38.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248**, 705–711.

Mazel, D., Dychino, B., Webb, V.A. and Davies, J. (1998). A distinct class of integron in the *Vibrio cholerae* genome. *Science* **280**, 605–608.

Mecsas, J. and Strauss, E.J. (1996). Molecular mechanisms of bacterial virulence: type III secretion and pathogenicity islands. *Emerg. Infect. Dis.* **2**, 271–288.

Mekalanos, J.J. (1983). Duplification and amplification of toxin genes in *Vibrio cholerae*. *Cell* **35**, 253–263.

Mellies, J.L., Navarro-Garcia, F., Okeke, I., Frederickson, J., Nataro, J.P. and Kaper, J.B. (2001). *espC* pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Infect. Immun.* **69**, 315–324.

Ménard, R., Sansonetti, P.J. and Parsot, C. (1994). The secretion of the *Shigella flexneri* Ipa invasins is induced by the epithelial cell and controlled by IpaB and IpaD. *EMBO J.* **13**, 555–568.

Middendorf, B., Hochhut, B., Leipold, K., Dobrindt, U., Blum-Oehler, G. and Hacker, J. (2004). Instability of pathogenicity islands in uropathogenic *Escherichia coli* 536. *J. Bacteriol.* **186**, 3086–3096.

Mikesell, P., Ivins, B.E., Ristroph, J.D. and Dreier, T.M. (1983). Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.* **39**, 371–376.

Miller, V.L. and Mekalanos, J.J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. *Proc. Natl. Acad. Sci. USA* **81**, 3471–3475.

Minton, N.P. (1995). Molecular genetics of clostridial neurotoxins. *Curr. Top. Microbiol. Immunol.* **195**, 161–194.

Miyoshi-Akiyama, T., Zhao, J., Kato, H., Kikuchi, K., Totsuka, K., Kataoka, Y., Katsumi, M. and Uchiyama, T. (2003). *Streptococcus dysgalactiae*-derived mitogen (SDM), a novel bacterial superantigen: characterization of its biological activity and predicted tertiary structure. *Mol. Microbiol.* **47**, 1589–1599.

Moncrief, J.S., Duncan, A.J., Wright, R.L., Barroso, L.A. and Wilkins, T.D. (1998). Molecular characterization of the fragilysin pathogenicity islet of enterotoxigenic *Bacteroides fragilis*. *Infect. Immun.* **66**, 1735–1739.

Müller, D., Hughes, C. and Goebel, W. (1983). Relationship between plasmid and chromosomal hemolysin determinants in *Escherichia coli*. *J. Bacteriol.* **153**, 846–851.

Nakagawa, I., Kurokawa, K., Yamashita, A., Nakata, M., Tomiyasu, Y., Okahashi, N., Kawabata, S., Yamazaki, K., Shiba, T., Yasunaga, T., Hayashi, H., Hattori, M. and Hamada, S. (2003). Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a

large-scale genomic rearrangement in invasive strains and new insights into phage evolution. *Genome Res*. **13**, 1042–1055.

Nakayama, K., Kanaya, S., Ohnishi, M., Terawaki, Y. and Hayashi, T. (1999). The complete nucleotide sequence of phi CTX, a cytotoxin-converting phage of *Pseudomonas aeruginosa*: implications for phage evolution and horizontal gene transfer via bacteriophages. *Mol. Microbiol*. **31**, 399–419.

Narita, S., Kaneko, J., Chiba, J., Piemont, Y., Jarraud, S., Etienne, J. and Kamio, Y. (2001). Phage conversion of Panton-Valentine leukocidin in *Staphylococcus aureus*: molecular analysis of a PVL-converting phage, phiSLT. *Gene* **268**, 195–206.

Nataro, J.P., Maher, K.O., Mackie, P. and Kaper, J.B. (1987). Characterization of plasmids encoding the adherence factor of enteropathogenic *Escherichia coli. Infect. Immun*. **55**, 2370–2377.

Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli*. Clin. *Microbiol. Rev*. **11**, 142–201.

Nataro, J.P., Seriwatana, J., Fasano, A., Maneval, D.R., Guers, L.D., Noriega, F., Dubovsky, F., Levine, M.M. and Morris, R.G., Jr (1995). Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *E. coli* and *Shigella* strains. *Infect. Immun*. **63**, 4721–4728.

O'Brien, A.D., Tesh, V.L., Donohue-Rolfe, A., Jackson, M.P., Olsnes, S., Sandvig, K., Lindberg, A.A. and Keusch, G.T. (1992). Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Curr. Top. Microbiol. Immunol*. **180**, 65–94.

Ochman, H., Lawrence, J.G. and Groisman, E.A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**, 299–304.

Ochman, H. and Moran, N.A. (2001). Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science* **292**, 1096–1099.

Okinaka, R.T., Cloud, K., Hampton, O., Hoffmaster, A.R., Hill, K.K., Keim, P., Koehler, T.M., Lamke, G., Kumano, S., Mahillon, J., Manter, D., Martinez, Y., Ricke, D., Svensson, R. and Jackson, P.J. (1999). Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *J. Bacteriol*. **181**, 6509–6515.

Oswald, E. and De Rycke, J. (1990). A single protein of 110 kDa is associated with the multinucleating and necrotizing activity encoded by the Vir plasmid of *Escherichia coli. FEMS Microbiol. Lett*. **56**, 279–284.

Oswald, E., Sugai, M., Labigne, A., Wu, H.C., Fiorentini, C., Boquet, P. and O'Brien, A.D. (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl. Acad. Sci. USA* **91**, 3814–3818.

Parreira, V.R. and Gyles, C.L. (2003). A novel pathogenicity island integrated adjacent to the thrW tRNA gene of avian pathogenic *Escherichia coli* encodes a vacuolating autotransporter toxin. *Infect. Immun*. **71**, 5087–5096.

Parsot, C. and Sansonetti, P.J. (1996). Invasion and the pathogenesis of *Shigella* infections. *Curr. Top. Microbiol. Immunol*. **209**, 25–42.

Paton, A.W., Paton, J.C., Heuzenroeder, M.W., Goldwater, P.N. and Manning, P.A. (1992). Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. *Microb. Pathog*. **13**, 225–236.

Paton, A.W., Paton, J.C., Goldwater, P.N., Heuzenroeder, M.W. and Manning, P.A. (1993). Sequence of a variant Shiga-like toxin type-I operon of *Escherichia coli* O111:H-. *Gene* **129**, 87–92.

Pearson, G.D.N., Woods, A., Chiang, S.L. and Mekalanos, J.J. (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. *Proc. Natl. Acad. Sci. USA* **90**, 3750–3754.

Peres, S.Y., Marches, O., Daigle, F., Nougayrede, J.P., Herault, F., Tasca, C., De Rycke, J. and Oswald, E. (1997). A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. *Mol. Microbiol*. **24**, 1095–1107.

Portnoy, D.A., Chakraborty, T., Goebel, W. and Cossart, P. (1992). Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun*. **60**, 1263–1267.

Portnoy, D.A. and Falkow, S. (1981). Virulence-associated plasmids from *Yersina enterocolitica* and *Yersinia pestis*. *J. Bacteriol*. **148**, 877–883.

Pullinger, G.D., Bevir, T. and Lax, A.J. (2004). The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. *Mol. Microbiol*. **51**, 255–269.

Rajamukar, J., Sasakawa, C. and Adler, B. (1997). Use of a novel approach, termed island probing, identifies the *Shigella flexneri she* pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. *Infect. Immun*. **65**, 4604–4614.

Rappuoli, R. and Pizza, M. (1991). Structure and evolutionary aspects of ADP-ribosylating toxins. In: *Sourcebook of Bacterial Protein Toxins* (eds J.E. Alouf and J.H. Freer), pp. 1-22, Academic Press, London.

Rappuoli, R. and Ratti, G. (1984). Physical map of the chromosomal region of *Corynebacterium diphtheriae* containing corynephage attachment sites *attB1* and *attB2*. *J. Bacteriol*. **158**, 325–330.

Rasko, D.A., Ravel, J., Okstad, O.A., Helgason, E., Cer, R.Z., Jiang, L., Shores, K.A., Fouts, D.E., Tourasse, N.J., Angiuoli, S.V., Kolonay, J., Nelson, W.C., Kolsto, A.B., Fraser, C.M. and Read, T.D. (2004). The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic Acids Res*. **32**, 977–988.

Ratti, G., Covacci, A. and Rappuoli, R. (1997). A $tRNA_2^{Arg}$ gene of *Corynebacterium diphtheriae* is the chromosomal integration site for toxinogenic bacteriophages. *Mol. Microbiol*. **25**, 1179–1181.

Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., Gill, S.R., Holtzapple, E.K., Okstad, O.A., Helgason, E., Rilstone, J., Wu, M., Kolonay, J.F., Beanan, M.J., Dodson, R.J., Brinkac, L.M., Gwinn, M., DeBoy, R.T., Madpu, R., Daugherty, S.C., Durkin, A.S., Haft, D.H., Nelson, W.C., Peterson, J.D., Pop, M., Khouri, H.M., Radune, D., Benton, J.L., Mahamoud, Y., Jiang, L., Hance, I.R., Weidman, J.F., Berry, K.J., Plaut, R.D., Wolf, A.M., Watkins, K.L., Nierman, W.C., Hazen, A., Cline, R., Redmond, C., Thwaite, J.E., White, O., Salzberg, S.L., Thomason, B., Friedlander, A.M., Koehler, T.M., Hanna, P.C., Kolsto, A.B. and Fraser, C.M. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423**, 81–86.

Rowe-Magnus, D.A. and Mazel, D. (2001). Integrons: natural tools for bacterial genome evolution. *Curr. Opin. Microbiol*. **4**, 565–569.

Sako, T. and Tsuchida, N. (1983). Nucleotide sequence of the staphylokinase gene from *Staphylococcus aureus*. *Nucleic Acids Res*. **11**, 7679–7693.

Sasakawa, C., Adler, B., Tobe, T., Okada, N., Nagai, S., Komatsu, K. and Yoshikawa, M. (1989). Functional organization and nucleotide sequence of virulence region-2 on the large virulence plasmid of *Shigella flexneri* 2a. *Mol. Microbiol*. **3**, 1191–1201.

Savarino, S.J., Fasano, A., Robertson, C. and Levine, M.M. (1991). Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. *J. Clin. Invest*. **87**, 1450–1455.

Savarino, S.J., Fasano, A., Watson, J., Martin, B.M., Levine, M.M., Guandalini, S. and Guerry, P. (1993). Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another

subfamily of *E. coli* heat-stable toxin. *Proc. Natl. Acad. Sci. USA* **90**, 3093–3097.

Savarino, S.J., McVeigh, A., Watson, J., Molina, J., Cravioto, A., Echeverria, P., Bhan, M.K., Levine, M.M. and Fasano, A. (1996). Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli. J. Inf. Dis.* **173**, 1019–1022.

Schmidt, H. and Karch, H. (1996). Enterohemolytic phenotypes and genotypes of Shiga toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic uremic syndrome. *J. Clin. Microbiol*. **34**, 2364–2367.

Schmidt, H., Beutin, L. and Karch, H. (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL933. *Infect. Immun*. **63**, 1055–1061.

Schmidt, H., Kernbach, C. and Karch, H (1996). Analysis of the EHEC *hly* operon and its location in the physical map of the large plasmid of enterhemorrhagic *Escherichia coli* O157:H7. *Microbiology*. **142**, 907–914.

Schmidt, H., Montag, M., Bockemühl, J., Heesemann, J. and Karch, H. (1993). Shiga-like toxin II-related cytotoxins *in Citrobacter freundii* strains from humans and beef samples. *Infect. Immun*. **61**, 534–543

Sears, C.L. and Kaper, J.B. (1996). Enteric bacterial toxins: mechanims of action and linkage to intestinal secretion. *Microbiol. Rev*. **60**, 167–215.

Shankar, N., Baghdayan, A.S. and Gilmore, M.S. (2002). Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis. Nature* **417**, 746–750.

Shankar, N., Coburn, P., Pillar, C., Haas, W. and Gilmore, M. (2004). Enterococcal cytolysin: activities and association with other virulence traits in a pathogenicity island. *Int. J. Med. Microbiol*. **293**, 609–618.

Sharp, P.M. (1991). Determinants of DNA sequence divergence between *Escherichia coli* and *Salmonella typhinurium*: codon usage, map position, and concerted evolution. *J. Mol. Evol*. **33**, 23–33.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. and Hayashi, H. (2002). Complete genome sequence of *Clostridium perfringens*, an anerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* **99**, 996–1001.

Sixma, T.K., Kalk, K.H., van Zanten, B.A., Dauter, Z., Kingma, J., Witholt, B. and Hol, W.G. (1993). Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol*. **230**, 890–918.

Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Ricklefs, S.M., Porcella, S.F., Parkins, L.D., Beres, S.B., Campbell, D.S., Smith, T.M., Zhang, Q., Kapur, V., Daly, J.A., Veasy, L.G. and Musser, J.M. (2002). Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proc. Natl. Acad. Sci. USA* **99**, 4668–4673.

So, M. and McCarthy, B.J. (1980). Nucleotide sequence of transposon Tn1681 encoding a heat-stable toxin (ST) and its identification in enterotoxigenic *Escherichia coli* strains. *Proc. Natl. Acad. Sci. USA* **77**, 4011–4015.

Spangler, B.D. (1992). Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev*. **56**, 622–647.

Stathopoulos, C., Provence, D.L. and Curtiss, R., 3rd. (1999). Characterization of the avian pathogenic *Escherichia coli* hemagglutinin Tsh, a member of the immunoglobulin A protease-type family of autotransporters. *Infect. Immun*. **67,** 772–781.

Straley, S.C., Plano, G.V., Skrzypek, E. and Bliska, J.B. (1993). Yops of *Yersinia* spp. pathogenic for humans. *Infect. Immun*. **61,** 3105–3110.

Strom, M.S., Eklund, M.W. and Poysky, F.T. (1984). Plasmids in *Clostridium botulinum* and related *Clostridium* species. *Appl. Environ. Microbiol*. **48,** 956–963.

Sumby, P. and Waldor, M.K. (2003). Transcription of the toxin genes present within the Staphylococcal phage phiSa3ms is intimately linked with the phage's life cycle. *J. Bacteriol*. **185,** 6841–6851.

Swenson, D.L., Bukanov, N.O., Berg, D.E. and Welch, R.A. (1996). Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infect. Immun*. **64,** 3736–3743.

Takeda, Y. (1995). Shiga and Shiga-like (Vero) toxins, In: Bacterial toxins and virulence factors in disease, eds. J. Moss, B. Iglewski, M. Vaughan, and T.A.Tu, Marcel Dekker Inc., New York, pp. 313–326.

Tauschek, M., Strugnell, R.A., and Robins-Browne, R.M. (2002). Characterization and evidence of mobilization of the LEE pathogenicity island of rabbit-specific strains of enteropathogenic *Escherichia coli. Mol. Microbiol*. **44,** 1533–1550.

Thorne, C.B. (1985). Genetics of *Bacillus anthracis*. In: Microbiology-85, eds. L. Lieve, P.F. Bonventre, J.A. Morello, S. Schlessinger, S.D. Silver and H.C. Wu, ASM Press, Washington D.C., 56.

Toth, I., Herault, F., Beutin, L. and Oswald, E. (2003). Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). *J. Clin. Microbiol*. **41,** 4285–4291.

Travisano, M. and Inouye, M. (1995). Retrons: retroelements of no known function. *Trends Microbiol*. **3,** 209–211.

Ubeda, C., Tormo, M.A., Cucarella, C., Trotonda, P., Foster, T.J., Lasa, I. and Penades, J.R. (2003). Sip, an integrase protein with excision, circularization, and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity islands. *Mol. Microbiol*. **49,** 193–210.

Uchida, I., Sekizaki, T., Hashimoto, K. and Terakado, N. (1985). Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *J. Gen. Microbiol*. **131,** 363–367.

Venkatesan, M.M. and Buysse, J.M. (1991). Nucleotide sequence of invasion plasmid antigen gene *ipaA* from *Shigella flexneri* 5. *Nucleic Acids Res*. **18,** 1648.

Venkatesan, M.M., Buysse, J.M. and Kopecko, D.J. (1988). Characterization of invasion plasmid antigen (*ipaBCD*) genes from *Shigella flexneri. Proc. Natl. Acad. Sci. USA* **85,** 9317–9321.

Waalwijk, C., de Graaff, J. and MacLaren, D.M. (1984). Physical mapping of hemolysin plasmid pCW2, which codes for virulence of a nephropathogenic *Escherichia coli* strain. *J. Bacteriol*. **159,** 424–426.

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272,** 1910–1914.

Waterfield, N.R., Daborn, P.J. and ffrench-Constant, R.H. (2002). Genomic islands in *Photorhabdus. Trends Microbiol*. **10,** 541–545.

Warren, R., Rogolsky, M., Wiley, B.B. and Glasgow, L.A. (1975). Isolation of extrachromosomal deoxyribonucleic acid for exfoliative toxin production from phage group II *Staphylococcus aureus. J. Bacteriol*. **122,** 99–105.

Weiss, A.A., Johnson, F.D. and Burns, D.L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA* **90,** 2970–2974.

Welch, R.A., Burland, V., Plunkett, G., 3rd, Redford, P., Roesch, P., Rasko, D., Buckles, E.L., Liou, S.R., Boutin, A., Hackett, J., Stroud, D., Mayhew, G.F., Rose, D.J., Zhou, S., Schwartz, D.C., Perna, N.T., Mobley, H.L., Donnenberg, M.S. and Blattner, F.R. (2002). Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli. Proc. Natl. Acad. Sci. USA* **99,** 17020–17024.

Weeks, C.R. and Ferretti, J.J. (1984). The gene for type A streptococcal exotoxin (erythrogenic toxin) is located in bacteriophage T12. *Infect. Immun*. **46,** 531–536.

Whelan, S.M., Garcia, J.L., Elmore, M.J. and Minton, N.P. (1994). The botulinum neurotoxin gene of the type A *Clostridium botulinum* strain NCTC 2916 is followed by a gene (*lycA*) encoding a lysozyme. *Zentralbl. Bakteriol. [Suppl.]* **24,** 162–163.

Yahr, T.L., Goranson, J. and Frank, D.W. (1996). Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol. Microbiol*. **22,** 991–1103.

Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M., Nakayama, K., Yamada, S., Komatsuzawa, H. and Sugai, M. (2000). Phage conversion of exfoliative toxin A production in *Staphylococcus aureus. Mol. Microbiol*. **38,** 694–705.

Yamaguchi, T., Hayashi, T., Takami, H., Ohnishi, M., Murata, T., Nakayama, K., Asakawa, K., Ohara, M., Komatsuzawa, H. and Sugai, M. (2001). Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infect. Immun*. **69,** 7760–7771.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M. and Sugai, M. (2002). Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect. Immun*. **70,** 5835–5845.

Yamamoto, T. and Echeverria, P. (1996). Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E. coli* strains pathogenic for humans. *Infect. Immun*. **64,** 1441–1445.

Yarwood, J.M., McCormick, J.K., Paustian, M.L., Orwin, P.M., Kapur, V. and Schlievert, P.M. (2002). Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. Implications for the evolution of staphylococcal pathogenicity islands. *J. Biol. Chem*. **277,** 13138–13147.

Zagaglia, C., Casalino, M., Colonna, B., Conti, C., Calconi, A. and Nicoletti, M. (1991). Virulence plasmids of enteroinvasive *Escherichia coli* and *Shigella flexneri* integrate into a specific site on the host chromosome: integration greatly reduces expression of plasmid-carried virulence genes. *Infect. Immun*. **59,** 792–799.

Zhang, S., Iandolo, J.J. and Stewart, G.C. (1998). The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol. Lett*. **168,** 227–233.

Zhang, W., Bielaszewska, M., Kuczius, T. and Karch, H. (2002). Identification, characterization, and distribution of a Shiga toxin 1 gene variant (stx(1c)) in *Escherichia coli* strains isolated from humans. *J. Clin. Microbiol*. **40,** 1441–1446.

CHAPTER

# 4

# Regulation systems of toxin expression

*Camille Locht, Didier Lereclus, Julian I. Rood, and Bénédicte Fournier*

## INTRODUCTION

As important virulence factors, the production of bacterial protein toxins is generally tightly regulated (see Table 4.1). Their genes are often members of regulons that include additional genes encoding other virulence determinants, such as adhesins, invasins, iron uptake systems, and many others. These regulons are usually controlled at the transcriptional level by virtue of repression and/or transactivation via specific regulatory proteins. In some cases, the regulons are part of a complex regulatory network involving several activator or repressor proteins that may act in cascade. In this chapter, we have chosen to describe four different examples of the regulation of toxin production. As an example of regulation via a transcriptional activator, the *Bacillus cereus* PlcR regulon is presented. Regulation via a two-component system is exemplified by the *Bordetella pertussis* BvgA/S system, and complex networks involving several different regulatory systems are described using the examples of *Clostridium* and *Staphylococcus aureus*.

## REGULATION OF VIRULENCE GENE EXPRESSION IN *BACILLUS CEREUS*

The genus *Bacillus*, first described in 1872 by Ferdinand Cohn, includes a large number of Gram-positive bacterial species, all of which form resistant spores when they encounter unfavorable growth conditions. *Bacillus* spores are ubiquitous and are common in soil; however, not all *Bacillus* species are able to grow in this environment. Members of the genus *Bacillus* colonize various ecological niches including plants, insects, and mammals, depending on their properties. The genetics of *Bacillus* are very well known due to the extensive studies performed on *Bacillus subtilis* in recent decades (Sonenshein *et al.*, 1993, 2002).

The *Bacillus cereus* group includes a large number of bacteria distributed in six highly related species: *Bacillus anthracis*, *Bacillus cereus* (*sensu stricto*), *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, and *Bacillus weihenstephanensis* (Lechner *et al.*, 1998; Nakamura, 1994; Priest *et al.*, 1994). These species were originally determined on the basis of characteristic features such as the presence of a capsule and toxins (*B. anthracis*), the formation of rhyzoid colonies (*B. mycoides* and *B. pseudomycoides*), the presence of a parasporal crystal (*B. thuringiensis*), and psychrotolerance (*B. weihenstephanensis*).

The *B. anthracis* genes specifying the capsule and the toxic factors responsible for anthrax are located on two plasmids, pXO1 and pXO2. The transcription of these genes is activated by the regulator AtxA during vegetative multiplication. The genes encoding the insecticidal toxins of *B. thuringiensis* are located on large conjugative plasmids and are expressed during the stationary phase or during sporulation. The roles and expression patterns of these *B. anthracis*- and *B. thuringiensis*-specific toxin genes have been extensively studied and reviewed (Agaisse and Lereclus, 1995; Koehler, 2002; Mock and Fouet, 2001; Schnepf *et al.*, 1998).

**TABLE 4.1** Main genetic regulatory systems of bacterial toxin production

| Species | Toxins regulated | Production phase | Regulatory system | Environmental factors | Reference |
|---|---|---|---|---|---|
| | | | **Two-component system** | | |
| *Bordetella pertussis* | Pertussis toxin<br>Adenylate cyclase toxin<br>Dermonecrotic toxin | exponential growth | BvgS/BvgA | Temperature<br>Nicotinic acid<br>$MgSO_4$ | (Stibitz, 2003) |
| *Clostridium perfringens* | Perfringolysin O<br>Alpha-toxin<br>Collagenase<br>Beta2 toxin | exponential growth | VirS/VirR<br>VirS/VirR-dependent VR-RNA | | (Rood, 1998) |
| *Staphylococcus aureus* | Haemolysins<br>Enterotoxins<br>Toxic shock syndrome toxin<br>Exfoliative toxins<br>Proteases | Post-exponential growth | AgrA/AgrC<br>AgrA/AgrC-dependent regulated RNA (RNAIII) | | |
| *Streptococcus pyogenes* | Streptolysin S<br>Streptokinase<br>Mitogenic factor | exponential growth | CovS/CovR | | (Stibitz, 2003) |
| | | | **Iron-dependent regulators** | | |
| *Corynebacterium diphtheriae* | Diphtheria toxin (DT) | exponential growth | DT regulator (DtxR, iron-binding repressor) | Iron | (Payne, 2003) |
| *Shigella dyssenteriae* | Shiga toxin | | Fur (Iron-binding repressor) | | |
| *Escherichia coli* | Haemolysin | | Fur | | |
| *Pseudomonas aeruginosa* | Exotoxin A | | Fur<br>RegA, PtxR | Iron, $O_2$, temperature, .... | |
| | | | **AraC family regulators** | | |
| *Pseudomonas aeruginosa* | Type III-secreted toxins (ExoU, ExoS, ExoT, Exo Y) | Exponential growth | ExsA | | (Gallegos *et al.*, 1997) |
| | | | **Cascade of activators, repressors**<br>**Pleiotropic activator** | | |
| *Vibrio cholerae* | Cholera toxin | exponential growth | ToxR, ToxS, ToxT | pH, temperature, osmolarity, aeration, amino acids temperature, $CO_2$ | (Hirst, 1999) (see chapter 19) |
| *Bacillus anthracis* | Lethal toxin<br>Edema toxin | exponential growth | AtxA, AcpA, AtxR | | (Leppla, 2000) |
| *Bacillus thuringiensis*, *Bacillus cereus* | Insecticidal toxins | | PlcR | | (Lereclus *et al.*, 2000) |
| | | | **Alternative sigma factors**<br>***sporulation-dependent alternative sigma factors*** | | |
| *Clostridium perfringens* | Enterotoxin | sporulation phase | SigK, SigE | | (Rood, 1998) |

*(continued)*

**TABLE 4.1** Main genetic regulatory systems of bacterial toxin production—cont'd

| Species | Toxins regulated | Production phase | Regulatory system | Environmental factors | Reference |
|---|---|---|---|---|---|
| | | | ***Transition phase alternative sigma factors*** | | |
| *Clostridium difficile* | Toxin A, toxin B | end of exponential growth-early stationary phase | TcdR | glucose, biotin, temperature | |
| *Clostridium perfringens* | Bacteriocin | | UviA | ? | (Raffestin *et al.*, 2004) |
| *Clostridium botulinum* | Botulinum neurotoxins | | BotR | ? | |
| *Clostridum tetani* | Tetanus toxin | | TetR | short peptides | |
| | | | **Qurum sensing system** | **Autoinducer** | |
| *Pseudomonas peruginosa* | Exotoxin A Proteases | | LasR, LasI | *N*-(3-oxoddo-decanoy | (Miller and Bassler, 2001) |
| | Many other genes | Rh1R, RhlI | 1-L-homoserine lactone | *N*-butyril-L-homoserine lactone | |

Besides these species-specific traits, members of the *B. cereus* group also share a large pool of genes, which constitutes a common genetic background representing about 75% of their genomes (Ivanova *et al.*, 2003; Kolstø *et al.*, 2002; Rasko *et al.*, 2004). Several of these genes encode extracellular compounds that might contribute to the virulence of *B. cereus* and *B. thuringiensis*. These compounds are degradative enzymes (phospholipases C, proteases, collagenases, etc.), cytotoxic proteins (hemolysins, enterotoxins, and cytotoxins), and cell surface proteins. Here, we briefly review the transcriptional mechanisms that control the expression of these virulence factors thought to be involved in gastrointestinal or nongastrointestinal infections.

## PlcR, a pleiotropic transcriptional activator

The PlcR regulator was first identified in *B. thuringiensis*, in which it was found to be required for transcription of the *plcA* gene, which encodes phosphatidylinositol-specific phospholipase C (PI-PLC) (Lereclus *et al.*, 1996). PlcR activates its own transcription and that of *plcA* at the end of the exponential growth phase. This regulator is a 34-kDa polypeptide, which contains a helix-turnhelix DNA-binding domain at its amino-terminal end. Analysis of the molecule reveals that PlcR contains structural motifs related to tetratricopeptide repeats (TPRs), which are domains putatively involved in protein-protein interaction (Perego and Hoch, 2002).

A genetic screening approach was used to detect *B. thuringiensis* genes regulated by PlcR (Agaisse *et al.*, 1999). This revealed that PlcR is a pleiotropic regulator that controls the transcription of several genes encoding exported proteins putatively involved in virulence, including the hemolytic and non-hemolytic enterotoxins, Hbl and Nhe. Alignment of the promoter regions activated by PlcR revealed a highly conserved palindromic sequence (TATGNANNNNANCATA). The –10 region of the PlcR-regulated promoters resembles that of promoters bound by an RNA polymerase associated with the sigma A factor. The –35 region of these promoters can significantly diverge from the –35 sequence generally recognized by sigma A. The transcriptional start site of the genes positively regulated by PlcR can be located at various positions downstream of the palindromic sequence (from a few nucleotides up to about 200 nucleotides in the case of the *hbl* and *nhe* operons).

Genetic experiments and DNase I footprinting have demonstrated that this 16-bp palindromic sequence (called the *PlcR box*) is the specific recognition site for PlcR (Slamti and Lereclus, 2002). However, analysis of two PlcR-regulated genes, encoding the metalloprotease InhA2 and the cytotoxin CytK, indicated that mutations in the consensus sequence do not necessarily affect PlcR activation (Brillard and Lereclus, 2004; Fedhila *et al.*, 2003). A recent study in which the PlcR box was subject to site-directed mutagenesis showed

that modification of the first or last nucleotide of the 16-bp palindrome does not drastically reduce PlcR activation (M. Gominet and D. Lereclus, unpublished data).

PlcR is active in *B. cereus* and *B. thuringiensis*, and most of the PlcR-regulated genes are found in both species (Agaisse *et al.*, 1999; Ivanova *et al.*, 2003; Økstad *et al.*, 1999). The PlcR-regulated genes are scattered throughout the chromosome rather than being clustered in a pathogenicity island. However, they tend to be arranged in pairs of genes that are transcribed in opposite orientations from a single, central PlcR box.

## Role and size of the PlcR regulon in *B. cereus* and *B. thuringiensis*

*In silico* analysis, based on the published PlcR box (TATGNANNNNTNCATA), suggested the presence of about 100 PlcR-regulated genes in the *B. cereus* genome (Ivanova *et al.*, 2003; Rasko *et al.*, 2004). Although not exact due to the inaccuracy of the PlcR box sequence, this result reflects the importance of the PlcR regulon in *B. cereus*. This was also clearly shown by the comparison of the extracellular proteomes of a *B. cereus* wild-type strain and a Δ*plcR* mutant strain. It appears that about 80% of all extracellular proteins depend on PlcR (Gohar *et al.*, 2002). The following major genes (and their products) unambiguously belong to the PlcR regulon, based on experimental evidence (transcriptional or 2D-electrophoresis analysis): *plcA* (PI-PLC), *plcB* (PC-PLC), *cerB* (sphingomyelinase), *hblCDA* (enterotoxic hemolysin BL), *nheABC* (non-hemolytic enterotoxin), *cytK* (cytotoxin), *inhA2*, *nprB*, and *nprP2* (metalloproteases), *colB* (collagenase), *sfp* (protease), *clo* and *tlo* (cereolysin O and thuringiolysin O, in *B. cereus* and *B. thuringiensis*, respectively).

The properties of the proteins encoded by the PlcR-regulated genes (degradative enzymes and toxins) suggest that the PlcR regulon plays an important role in the adaptation of the bacteria to their environment and in the virulence of the bacteria in susceptible hosts. Deletion of the *plcR* gene in *B. thuringiensis* and *B. cereus* drastically reduces their virulence in orally infected insects, in mice infected by nasal instillation, and in endophthalmitis (Callegan *et al.*, 2003; Salamitou *et al.*, 2000). The precise role of each PlcR-regulated gene in pathogenicity has not been determined. However, a *B. cereus* strain overproducing CytK has been shown to cause severe gastroenteritis (Lund *et al.*, 2000), and the metalloprotease InhA2 is necessary for the virulence of *B. thuringiensis* in insects (Fedhila *et al.*, 2002; Fedhila *et al.*, 2003).

## The PlcR regulon is not functional in *B. anthracis* and in a few *B. cereus* strains

*B. anthracis* and about 1% of *B. cereus* and *B. thuringiensis* strains are devoid of hemolytic and lecithinase activities. In *B. anthracis*, a nonsense mutation in the *plcR* gene results in a truncated inactive product (Agaisse *et al.*, 1999). A nonsense mutation (mapping at a different nucleotide position to the mutation in *B. anthracis*), and deletion or point mutations have been identified in the *plcR* sequences of Hly$^-$, Lec$^-$ *B. cereus*, and *B. thuringiensis* strains (Slamti *et al.*, 2004). Introduction of the active *plcR* gene from *B. thuringiensis* strain 407 restored the hemolytic and lecithinase activities of these strains (Mignot *et al.*, 2001; Slamti *et al.*, 2004). Thus, the genes of the PlcR regulon are present and potentially functional in these strains. This also indicates that major distinctive traits of *B. anthracis* (*i.e.*, absence of hemolytic, lecithinase, and protease activities) result from a single mutation in a pleiotropic regulator. However, for unknown reasons, the introduction of the active *plcR* gene from *B. cereus* strain 569 does not restore hemolytic and lecitinase activities in *B. anthracis* (Pomerantsev *et al.*, 2003).

## Expression of the PlcR regulon is controlled by quorum sensing

A small PlcR-regulated gene, *papR*, located 70 bp downstream from *plcR*, is required for the expression of the PlcR regulon genes. Deletion of the *papR* gene abolishes the production of hemolysins and degradative enzymes, and results in a large decrease in virulence (Slamti and Lereclus, 2002). The *papR* gene product is secreted and diffusible in the extracellular medium. A processed form of PapR, corresponding to the carboxy-terminal end of the peptide (which may contain as few as 5 residues), is subsequently imported into the bacterial cells. The oligopeptide permease Opp is necessary for *plcR* expression (Gominet *et al.*, 2001), suggesting that the uptake of the C-terminal part of PapR is ensured by Opp. Once inside the bacteria, the processed form of PapR (presumably a pentapeptide) interacts with PlcR to facilitate its binding to its DNA targets (Slamti and Lereclus, 2002). The mechanism leading to the PlcR-PapR interaction and to the consequent activation of PlcR is not known. However, a translational fusion between PlcR and PapR is functional, suggesting that the interaction between the two peptides involves the C-terminal part of PlcR (Pomerantsev *et al.*, 2004).

The activation of PlcR by PapR is strain-specific, and this specificity is determined by the first residue of the pentapeptide (Slamti and Lereclus, 2002). Alignment

of PapR sequences from various strains of the *B. cereus* group and PlcR activation assays using various PapR pentapeptides have led to the identification of specificity groups. Four types of pentapeptide (LPFEF, VPFEF, MPFEF, LPFEH) have been identified in the bacteria forming the *B. cereus* group. A strain producing the pentapeptide LEFEF is able to activate the PlcR regulon of a strain belonging to the same specificity group. However, this PlcR regulon is not activated by the strains producing the pentapeptides LPFEH, VPFEF, or MPFEF (Slamti and Lereclus, 2002, 2005). Hence, the bacteria belonging to a given PlcR-PapR group are able to produce simultaneously a set of virulence factors in response to the same molecular signal.

A general model for the regulation of PlcR expression is presented in Figure 4.1. First, this model assumes that low but constant amounts of PlcR and PapR are produced during the vegetative growth phase. At the onset of the stationary phase, the increase in the intracellular concentration of PapR leads to the activation of PlcR and consequently to the expression of the PlcR regulon genes. This regulatory system, which coordinates gene expression and cell density, is designated quorum-sensing. Second, in response to severe nutriment deprivation, the concentration of Spo0A~P increases in the bacterial cells and activates the sporulation process. Spo0A~P is also a repressor of *plcR* transcription (Lereclus *et al.*, 2000), and consequently the expression of the PlcR regulon is switched off or strongly reduced during sporulation. The expression of the PlcR regulon is thus controlled at both the transcriptional level (with Spo0A) and the post-transcriptional level (with PapR). This double regulation allows the bacteria to adapt to various parameters like cell density and nutriment availability. The selection and the maintenance of this regulatory system allows the bacteria of the *B. cereus* group to start the infection process when the environmental conditions are favorable and when, and only when, the number of bacteria is "estimated" to be sufficient to elicit infection.

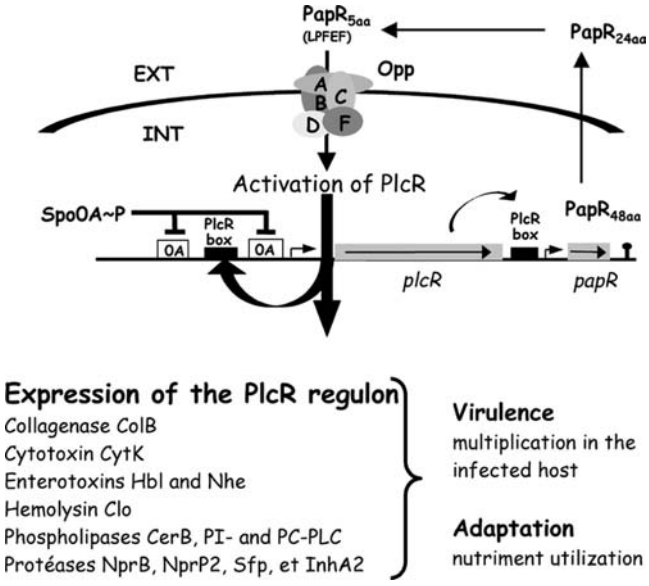



**FIGURE 4.1** Model for the regulation of PlcR expression.The *plcR* and *papR* genes are expressed at a low and basal level during the exponential growth phase. PapR (48 amino acids) is secreted into the extracellular medium, presumably via the SecA system. The exported peptide (24 amino acids) is then processed to give a smaller peptide, thought to be a pentapeptide. This small PapR peptide diffuses in the extracellular medium and is imported into the bacteria via Opp. Once inside the cell, the peptide interacts with PlcR, allowing it to bind the PlcR boxes. This binding activates the transcription of the PlcR regulon. When the bacteria encounter unfavorable environmental conditions (nutriment deprivation), the phosphorylation status of the protein Spo0A increases in the cells. As a consequence, Spo0A~P activates the sporulation process and blocks the transcription of *plcR*. Consequently, the expression of the PlcR regulon is switched off during sporulation.

## THE *BORDETELLA* BVGA/S SYSTEM

Many bacterial pathogens regulate the production of their virulence factors at the transcriptional level by signal-transducing two-component systems. In its minimal structure, these systems comprise a membrane-associated sensor protein and a cytoplasmic transcriptional activator (Stock *et al.*, 1989). Extracellular, but sometimes also intracellular signals, are sensed by the sensor protein. This information is then transmitted to the activator protein by a phosphorylation cascade that results in the activation of the transcription factor and, consequently, in the transcription, or sometimes repression, of the genes regulated by the two-component system.

### Phenotypic modulation and phase variation

The genes coding for the *Bordetella* protein toxins, as well as those encoding most of the other *Bordetella* virulence factors are under the control of the BvgA/S (for **B***ordetella* **v**irulence **g**ene) two-component system. BvgS represents the sensor component, and BvgA is the transcriptional activator. That the *Bordetella* virulence genes are coordinately regulated was suggested more than 40 years ago when Lacey (1960) reported that the *Bordetella pertussis* growth conditions strongly influence the overall virulence of the organism. When grown at 25°C or in the presence of $MgSO_4$ or of nicotinic acid, the virulence factors are collectively not

produced, and *B. pertussis* is in the C mode. In contrast, when grown at 37°C and in the absence of $MgSO_4$ and nicotinic acid, the organism is in the X mode, and the virulence factors are produced. This reversible phenomenon is termed *phenotypic modulation*. In addition to the reversible phenotypic modulation, it was already known in the early 1930s that *B. pertussis* could undergo essentially irreversible phase variation (Leslie and Gardner, 1931). Virulent (phase I) organisms can become avirulent (phase IV) at a frequency of $10^{-3}$ to $10^{-6}$, depending on the strain and on the culture conditions. Phase IV organisms are characterized by the loss of essentially all virulence factors.

The molecular mechanism of phase variation has been elucidated by Stibitz *et al.* (1989), who showed that the transition from phase I to phase IV in *B. pertussis* can be explained by the insertion or deletion of a cytosine in a string of six consecutive cytosines located in the coding sequence of a gene, subsequently called *bvgS*. In other *Bordetella* strains, in particular in *B. bronchiseptica*, other deletions or insertions in the *bvg* locus are responsible for phase variation (Monack *et al.*, 1989). Although the precise molecular mechanisms have not yet been identified, phenotypic modulation is probably due to the ability of BvgS to sense the different environmental conditions to which *Bordetella* can be exposed and to transmit this information via phosphorylation to BvgA.

Phenotypic modulation can readily be induced in laboratory conditions, but it is not clear yet what role it plays during natural infection, nor which modulatory signals are perceived within the host environment. However, it has been shown that the precise regulation of *Bordetella* virulence genes is essential for pathogenesis, as ectopic expression of the virulence genes results in loss of virulence (Akerley *et al.*, 1995). In addition to the $Bvg^+$ (phase I) and $Bvg^-$ (phase IV) phases, an intermediate phase has been identified for *B. bronchiseptica* (Cotter and Miller, 1997). This phase, named Bvgi, is characterized by the production of certain virulence factors, such as filamentous hemagglutinin and fimbriae, but not of others, such as the adenylate cyclase toxin. Furthermore, a set of Bvgi-specific antigens is produced by *B. bronchiseptica* during this intermediate phase. Recent studies suggest that this phase may be important for biofilm formation (Irie *et al.*, 2004). The signals sensed by the BvgA/S system may perhaps be related to the metabolic pathways and growth state of the bacterium. When grown at high density, sulfure-containing amino acids accumulate in the media, which can result in elevated $SO_4^{2-}$ release (Bogdan *et al.*, 2001). This finally can then result in BvgA/S-mediated modulation of *Bordetella* virulence gene expression.

## Structure-function relationship of BvgA and BvgS

Compared to classical two-component systems, the BvgA/S system presents an "unorthodox" modular structure. Whereas in its simplest form the sensor proteins are composed of two domains, the extracytoplasmic sensor domain and the cytoplasmic transmitter domain, BvgS contains two additional cytoplasmic domains. These additional domains comprise another receiver and another transmission domain (Uhl and Miller, 1994). All three cytoplasmic domains play a crucial role in phosphorylation-mediated signal transmission from BvgS to BvgA, as mutations in either module abolish activation of BvgA. The three cytoplasmic domains are separated by alanine/proline-rich sequences and participate in a phosphorylation cascade in which the first transmitter domain is autophosphorylated, and the receiver domain regulates the transfer of the phosphoryl group from the autophosphorylated transmitter to the C-terminal, second transmitter domain. Only the phosphorylated C-terminal transmitter domain is then able to transfer the phosphoryl group to BvgA. A similar modular structure and phosphorelay system has been found for the *Escherichia coli* drug efflux regulatory system EvgA/S (Eguchi *et al.*, 2003). However, despite the strong sequence similarities between BvgS and EvgS, these two proteins display a high degree of specificity for their cognate regulators, BvgA and EvgA, respectively, and this specificity is due to the C-terminal, second transmitter domain of these sensor proteins (Perraud *et al.*, 1998).

The N-terminal, periplasmic domain of BvgS is likely to be involved in environmental sensing. Exchange experiments between the *B. pertussis* and the *B. bronchiseptica* BvgS of discrete fragments have indicated that signal sensitivity, such as the concentration of $MgSO_4$ required for phenotypic modulation, depends on the N-terminal, periplasmic domain of BvgS (Martinez de Tejada *et al.*, 1996). However, a short linker region, located between the transmembrane and the cytoplasmic domains of BvgS, appears also to be involved in environmental sensing, as amino acid substitutions in this linker region may cause insensitivity to modulating agents, such as nicotinic acid (Goyard *et al.*, 1994; Manetti *et al.*, 1994). How exactly these two regions operate during signal transduction is not yet known. It is possible that they are required for oligomerization of BvgS, as the isolated cytoplasmic domain alone is not able to oligomerize (Perraud *et al.*, 2000). The active form of BvgS has been shown by transcomplementation studies and by a dominant negative phenotype observed after overexpression of a

mutant *bvgS* allele to be oligomeric (Stibitz and Yang, 1991). In addition to the periplasmic domain and the linker region, the cytoplasmic domain of BvgS may also constitute a target site of the sensor properties of the protein, as the kinase activity of BvgS has been shown to be very sensitive to the oxidation status of ubiquinone. Oxidized ubisuinone is a strong inhibitor of the kinase activity, whereas the reduced form of the quinone has no effect on the enzymatic activity (Bock and Gross, 2002), indicating that the BvgA/S system is also sensitive to the oxidation status of the cell.

The transfer of the phosphoryl group from the C-terminal domain of BvgS to the N-terminal receiver domain of BvgA results in activation of the transcription factor. The C-terminal, output domain of phosphorylated BvgA, is then able to bind specifically to the promoter regions of the *Bordetella* virulence genes (Boucher *et al.*, 1994). Phosphorylation of BvgA enhances DNA binding, although unphosphorylated BvgA may still bind to a subset of *bvg*-regulated promoters. DNA binding alone is not sufficient for transactivation. Transactivation requires synergistic binding of RNA polymerase and phosphorylated BvgA to the DNA target sites (Boucher and Stibitz, 1995). The interaction of BvgA with RNA polymerase involves the alpha subunit of the polymerase (Stibitz, 1998), and in particular its C-terminal domain (Carbonetti *et al.*, 2000). The minimal functional BvgA unit is probably a dimer. Detailed analyses of one of the *bvg*-regulated promoters have shown that, at least for the *fha* promoter, three dimers, formed by head-to-head association of the monomers, bind to one face of the DNA helix, whereas the C-terminal domain of the RNA polymerase alpha subunit binds to the opposite face of the helix (Boucher *et al.*, 2003). This is then believed to result in the initiation of transcription of the target genes.

## The BvgA/S regulon

Most of the identified virulence genes of *Bordetella* are activated by the BvgA/S system and are collectively called *vag*s (for ***v***irulence ***a***ctivated ***g***enes). They include the genes that encode the classical virulence factors, such as pertussis toxin (PTX), adenylate cyclase toxin (AC), dermonecrotic toxin, filamentous hemagglutinin (FHA), fimbriae, pertactin, tracheal colonization factor, Vag8, Brk, and type III secretion systems. The promoter structure and BvgA-binding sites of a limited set of *vag*s has been analyzed in detail, such as those of the *fha*, the *cya*, and *ptx* genes. Although a clear consensus BvgA-binding site has not been identified, studies on the *cya*, *bvg* (Karimova and Ullmann, 1997), and *ptx* promoters (Marques and Carbonetti, 1997) have shown that they consist of inverted repeats. However, in some promoter regions, the BvgA-binding sites may also contain direct repeats. They are generally located from 50 to 150 nucleotides upstream of the transcriptional start site and usually comprise multiple BvgA-binding sites, as has most extensively been demonstrated in the *fha* promoter region (Boucher *et al.*, 2003). The *fha* promoter is up to now probably the most well characterized *bvg*-dependent promoter (Boucher *et al.*, 2001a, b). Studies on the *fha* promoter have indicated that the nucleotides at positions 3, 4, and 7 of the BvgA-binding heptad are crucial for the binding of the transcription factor, and that the promoter contains both high- and low-affinity sites for phosphorylated BvgA, the latter of which shows limited DNA specificity (Boucher *et al.*, 2001a). Binding to the primary, high-affinity site and to the secondary, low-affinity region is cooperative.

Whereas BvgA can bind to some promoters in the unphosphorylated form, phosphorylation is essential for binding to other promoters, such as the *ptx* promoter, suggesting that differential binding of BvgA may govern different regulatory mechanisms of *bvg*-activated genes. Upon the induction of the *bvg*-activated genes, some genes such as *fha* are transcribed within minutes, while others such as *prn* are transcribed only after one hour, and others, such as *ptx*, require several hours before they are expressed (Kinnear *et al.*, 2001). The precise kinetics of activation of the individual genes appear to be critical for the expression of *B. pertussis* virulence in mouse models.

In addition to the classical virulence genes, the BvgA/S system may also regulate other genes, such as those that encode lipid A modifying enzymes (Preston *et al.*, 2003) and proteins involved in iron uptake (Passerini de Rossi *et al.*, 2003). The availability of the entire genome sequence (Parkhill *et al.*, 2003) combined with new tools, such as DNA microarrays (Hot *et al.*, 2003) makes it now possible to identify all the members of the Bvg regulon.

BvgA also regulates the production of a series of transcription factors, one of which, BvgR, represses a set of genes named *vrg*s (for *v*irulence *r*epressed *g*enes). BvgR is a 32-kDa protein that probably binds to the operator sites of the *vrg*s (Merkel *et al.*, 1998a, b). The role of the *vrg*s in pathogenesis is not clear. While a BvgR-deficient mutant is strongly affected in virulence (Merkel *et al.*, 1998b), suggesting that it is important to repress *vrg*s during the infectious cycle, the mutational inactivation of individual *vrg*s does not appear to affect *Bordetella* virulence (Martinez de Tejada *et al.*, 1998). These observations indicate that down-regulation of *vrg*s, but not their expression, is involved in pathogenesis.

## GENETIC REGULATION IN CLOSTRIDIA

Gene regulation in Clostridia is a complex topic with many subtle intricacies and variations, far too many to be included in a chapter of this broad nature. This section will therefore focus on giving an overview of the regulation of toxin production in four pathogenic clostridial species, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium botulinum*, and *Clostridium tetani*. It concentrates on three major mechanisms by which clostridial toxin gene expression is controlled, namely by two-component signal transduction, DNA bending, and by the use of alternative sigma factors.

### The global VirS/VirR two-component signal transduction system of C. *perfringens*

*C. perfringens* is the causative agent of clostridial myonecrosis (gas gangrene) and several gastrointestinal diseases. The essential toxin involved in clostridial myonecrosis is the α-toxin, with perfringolysin O (θ toxin) playing a secondary synergistic role (Titball and Rood, 2002). The VirS/VirS system regulates the production of α-toxin, perfringolysin O (θ-toxin), collagenase, and numerous housekeeping genes (Rood, 1998). It consists of the sensor histidine kinase, VirS, and its cognate response regulator, VirR. The regulatory cascade is triggered by an unknown growth phase or environmental signal, most likely a secreted peptide that is part of a classical Gram-positive quorum sensing system. Binding of the signal molecule to an external domain of the VirS transmembrane protein is proposed to lead to a change in VirS conformation and autophosphorylation of a conserved His residue in its cytoplasmic C-terminal domain. Phosphorylated VirS then interacts with the cytoplasmic VirR molecule, and the phosphate residue is then transferred to a conserved Asp residue in the N-terminal domain of VirR.

In *C. perfringens* strain 13, the sequenced paradigm strain used in most genetic studies, VirS-activated VirR has been shown to directly activate the transcription of five genes that are located at different sites in the genome (Ba-Thein *et al*., 1996; Shimizu *et al*., 2002a; Cheung *et al*., 2004). These genes include the perfringolysin O structural gene, *pfoA*, the α-clostripain cysteine protease gene, a gene encoding the regulatory RNA molecule, VR-RNA, and two genes of unknown function (Rood, 1998; Banu *et al*., 2000; Shimizu *et al*., 2002b). Each of these genes has two 13 bp imperfect direct repeats, or VirR boxes, located just upstream of the -35 box of the promoter (Shimizu *et al*., 2002a).

VirR binds independently to each VirR box (Cheung and Rood, 2000), but both VirR boxes are essential for transcriptional activation of the downstream promoter (Cheung *et al*., 2004). Both the spacing between the VirR boxes and the distance between these boxes and the -35 box are important for biological activity. Experimental evidence indicates that VirR binding is required for the binding of *C. perfringens* RNA polymerase to the promoter (Cheung *et al*., 2004). Other studies have shown that the FxRxHrS and SKHR motifs, which are located in the C-terminal activator domain of VirR, are required for DNA binding and biological activity (McGowan *et al*., 2002; McGowan *et al*., 2003).

VirR also activates other toxin and housekeeping genes indirectly, as a result of its ability to activate the transcription of the VR-RNA molecule (Banu *et al*., 2000; Shimizu *et al*., 2002c). The VirS/VirR regulon therefore represents an extensive regulatory cascade that is involved in more than toxin gene regulation; even plasmid-encoded genes can be regulated by this system (Ohtani *et al*., 2003). The VR-RNA molecule partially activates transcription of the α-toxin structural gene, *plc*, the collagenase gene, *colA*, the beta-2 toxin gene *cbp2*, which is encoded on the cryptic plasmid pCP13, a cyclic nucleotide phosphodiesterase gene, *cpd*, the *ptp* gene, and other genes identified from proteomic studies (Shimizu *et al*., 2002b). In addition, it negatively regulates the transcription of an operon involved in cysteine metabolism and the pCP13-determined *cna* gene. The mechanism by which VR-RNA mediates these pleiotropic effects is not known, but it is likely to involve a specific VR-RNA-binding protein. The elucidation of the mechanism of action of VR-RNA and the analysis of the signaling molecule that triggers the VirS/VirR cascade represent major challenges for researchers in this field.

### DNA curvature and α-toxin production in C. *perfringens*

The expression of the major *C. perfringens* extracellular toxin, the α-toxin, is down-regulated at temperatures greater than 20°C, which is consistent with the concept that this toxin has a major degradative role in the normal soil environment. Temperature-dependent regulation is mediated not by the VR-RNA molecule but by DNA bending induced by three phased poly(A)$_{5\text{-}6}$ tracts located upstream of the *plc* promoter (Toyonaga *et al*., 1992; Matsushita *et al*., 1996). Because of the presence of these poly(A) sequences, DNA curvature or bending is more pronounced at lower temperatures, resulting in higher *plc* expression (Katayama *et al*., 1999). Reduction of the number of poly(A) tracts

reduces the amount of DNA bending, with a concomitant reduction in *plc* expression.

Gel retardation and footprinting studies showed that poly(A) tract-mediated DNA bending stimulates the formation of a more stable RNA polymerase – $P_{plc}$ complex and leads to increased transcriptional activity. Subsequent studies showed that the C-terminal domain of the α-subunit of *C. perfringens* RNA polymerase binds to the minor grooves of the poly(A) tracts with increased binding being observed at lower temperatures (Katayama *et al*., 2001). This regulatory system is not restricted to the *plc* gene in *C. perfringens* since a similar biological effect is observed for the *C. perfringens* ferredoxin gene, *fdx*, which also has three phased poly(A) tracts upstream of the -35 box of the promoter, in addition to an additional two tracts located within the promoter (Kaji *et al*., 2003).

### Alternative sigma factors and toxin production

In recent years it has been shown that alternative sigma factors play an important regulatory role in the control of toxin production, particularly in *C. difficile*, *C. botulinum*, and *C. tetani*. These proteins include BotR, which regulates botulinum neurotoxin (BoNT) expression, TetR, which controls tetanus neurotoxin (TeNT) production, and TcdR (previously called TxeR or TcdD) (Rupnik *et al*., 2005), the toxin A and toxin B regulator from *C. difficile*. These proteins, together with the bacteriocin regulator UviA from *C. perfringens*, form a novel family of alternative sigma factors.

Botulinum toxin is released from the cell in the form of a complex that contains BoNT and several non-toxic proteins, including a hemagglutinin (Johnson, 2000). These proteins are all encoded by a contiguous gene locus that includes the *bont* toxin gene and the *botR* regulatory gene. Although the precise location and transcriptional orientation of *botR* varies in the different *C. botulinum* toxin types, it is always located within this locus, upstream of the *ntnh* and *bont* genes. Overexpresson of the BotR protein leads to increased production of both BoNT and its associated proteins, a process that is mediated at the transcriptional level by the binding of BotR to the relevant promoters (Marvaud *et al*., 1998a). Partial inhibition of *botR* expression with antisense mRNA leads to decreased toxin production. There is a closely related gene, *tetR*, located upstream of the TeNT structural gene, *tetX* (Marvaud *et al*., 1998b). The TetR protein has 50–65% identity to the equivalent BotR proteins and, as expected, overexpression of TetR leads to increased -TeNT production, again as a result of transcriptional activation. The BotR protein was also able to activate *tetX* expression in *C. tetani*, but less efficiently.

More recent studies have shown that BotR and TetR, like TcdR and UviA, are alternative sigma factors rather than positive regulators (Raffestin *et al*., 2005). They require RNA polymerase core enzyme to bind to the promoter region and to catalyze transcription *in vitro*. Deletion of a helix-turn-helix motif located in BotR did not affect the ability of the sigma factor to bind the RNAP core, but eliminated the binding of the resultant holoenzyme to bind to the promoter. Although the use of alternative sigma factors is an excellent mechanism for ensuring that specific gene products are only produced when required, the signals that lead to the functional activation of these factors are not known.

Initial studies showed that the *tcdR* gene (then called *txeR*), which is located immediately upstream of the toxin B structural gene *tcdB,* could activate the expression of *C. difficile* toxin gene fusions in *E. coli* (Moncrief *et al*., 1997). The TcdR dependent promoters have been identified and shown to have similarity to the UviA-dependent promoter of the *C. perfringens bcn* gene (Dupuy and Sonenshein, 1998). Subsequently, it was shown that the TcdR protein was an alternative sigma factor, the first member of this clostridial protein family to be identified as a sigma factor (Mani and Dupuy, 2001). TcdR is required for the specific binding of *C. difficile* RNA polymerase to the *tcdA* and *tcdB* promoters and activates transcription from these promoters *in vitro*, in the presence of RNA polymerase. It has been shown that TcdR regulates toxin reporter gene expression in both *C. perfringens* (Dupuy and Sonenshein, 1998; Mani and Dupuy, 2001) and *C. difficile* (Mani *et al*., 2002); in both species this regulatory process is subject to catabolite repression by glucose. TcdR also regulates its own expression (Mani *et al*., 2002) in a temperature-dependent manner (Karlsson *et al*., 2003). It was proposed that TcdR was the prototype member of an alternative sigma factor family that also included BotR, TetR, and UviA (Mani and Dupuy, 2001). Subsequent studies have confirmed this proposal, and these four proteins are now considered a distinct subgroup of the extracytoplasmic (ECF) family of $\sigma^{70}$ factors (Dupuy *et al*., 2005; Raffestin *et al*., 2005).

## GENETIC REGULATION OF *STAPHYLOCOCCUS AUREUS* EXOTOXINS

*Staphylococcus aureus* is a major human pathogen that causes a variety of infections, ranging from superficial lesions to life-threatening diseases, due to the wide repertoire of toxins, exoenzymes, adhesins, and immune-modulating proteins that it produces. With

the exception of diseases caused by specific toxins, no single virulence factor has been found to be sufficient to cause a staphylococcal infection. Thus, staphylococcal infections are attributable to the coordinated actions of several different factors. Some of these proteins, including cytotoxins and exoenzymes, are secreted. Others, including protein A and various adhesins, remain attached to the cell wall. The *Staphylococcus* virulence factors have been divided into three classes on the basis on their potential roles in the infectious process (Projan and Novick, 1997): (i) factors that allow the bacteria to attach to host cells or extracellular matrices; (ii) factors that allow them to evade the host defense system, i.e. factors that inhibit phagocytosis, and factors that decrease the activity of antistaphylococcal antibodies; and (iii) factors involved in invasion, i.e., factors that degrade cell or extracellular matrix components allowing dissemination of *S. aureus*.

*S. aureus* exotoxins can be divided into two general groups: (i) membrane-active agents or cytotoxins and (ii) toxins with superantigen activity.

- The membrane-active group contains α-, β-, and δ toxins, which are hemolytic, and bi-component toxins, which include leukocidins and have cytolytic effects on polymorphonuclear leukocytes, monocytes, and/or erythrocytes. The membrane-active toxins participate in the invasion phase of infection (Bronner *et al.*, 2004).
- One subset of staphylococcal diseases results primarily from a single toxin exhibiting superantigen activity. Among these toxinoses, toxic shock syndrome is provoked mostly by toxic-shock syndrome toxin-1 (TSST-1) and less frequently by enterotoxins such as enterotoxin A (SEA), B (SEB), C (SEC), or D (SED). In addition, enterotoxins (A to E and G to R) cause staphylococcal food poisoning. Finally, the exfoliative (epidermolytic) toxins of *S. aureus* are responsible for staphylococcal scalded skin syndrome, which generally affects young children. These superantigen toxins are encoded by mobile genetic elements such as plasmids, bacteriophages, and pathogenicity islands (McCormick *et al.*, 2001).

Relatively little is known about the regulation of virulence gene expression during infection. However, in recent years, many groups have investigated the regulatory systems that control the expression of these genes *in vitro*. Various studies of virulence factor production by *S. aureus* in batch cultures have shown that most exoproteins, including exotoxins, are secreted in only the post-exponential phase of bacterial growth, whereas cell wall–associated virulence factors, such as protein A, are produced during logarithmic phase (Projan and Novick, 1997) (See Figure 4.2). Their production is controlled by a complex global regulatory network of genes, the expression of which *in vitro* is temporally and coordinately regulated and generally influenced, directly or indirectly, by environmental signals.

## Virulence regulators

Several global regulators regulate the production of virulence-associated exoproteins. Some of them are two-component systems and are sensitive to environmental signals.

### *The Agr system*

The *a*ccessory *g*ene *r*egulator (Agr) is one of the best-characterized global regulatory systems. The Agr system is a two-component system responding to quorum sensing (Ji *et al.*, 1995). The *agr* locus produces two distinct and divergent transcripts, RNAII and RNAIII, which are initiated from two promoters (P2 and P3, respectively) (Novick *et al.*, 1995) (Figure 4.3). RNAIII overlaps the *hld* open reading frame encoding the δ toxin, but RNAIII itself functions as the regulatory signal of the Agr system (Morfeldt *et al.*, 1995; Novick *et al.*, 1993). The main role of RNAIII is to regulate the transcription of target genes directly or indirectly (Novick *et al.*, 1993). The mechanism by which RNAIII modulates transcription is unknown. However, RNAIII up-regulates *hla* (encoding the α-toxin) expression at the transcriptional and translational levels (Novick *et al.*, 1993). Indeed, an increase in RNAIII induces α- and β-toxin gene expression under *agr* regulation (Recsei *et al.*, 1986). At the level of translation, RNAIII serves as an antisense RNA. *hla* mRNA contains a secondary structure probably blocking translation. RNAIII base pairs with sequences on *hla* mRNA upstream of the translational start site and releases the ribosome-binding site and start codon necessary for translation of the mRNA molecule (Morfeldt *et al.*, 1995).

The principal role of the RNAII transcript is to regulate the transcription of RNAIII. The RNAII transcript potentially encodes four proteins, AgrB, AgrD, AgrC, and AgrA. RNAIII and RNAII synthesis depends on each of the four proteins (Novick *et al.*, 1995) (Figure 4.3). This suggests that the *agr* locus belongs to an autocatalytic feedback system. AgrA and AgrC constitute the response regulator and histidine kinase components, respectively, of a two-component system (Lina *et al.*, 1998; Novick *et al.*, 1995). During the exponential growth phase, the *agrD*-encoded propeptide is processed by proteolytic digestion and the formation of a thioester bond resulting in an autoinducing peptide (AIP). It was subsequently demonstrated that

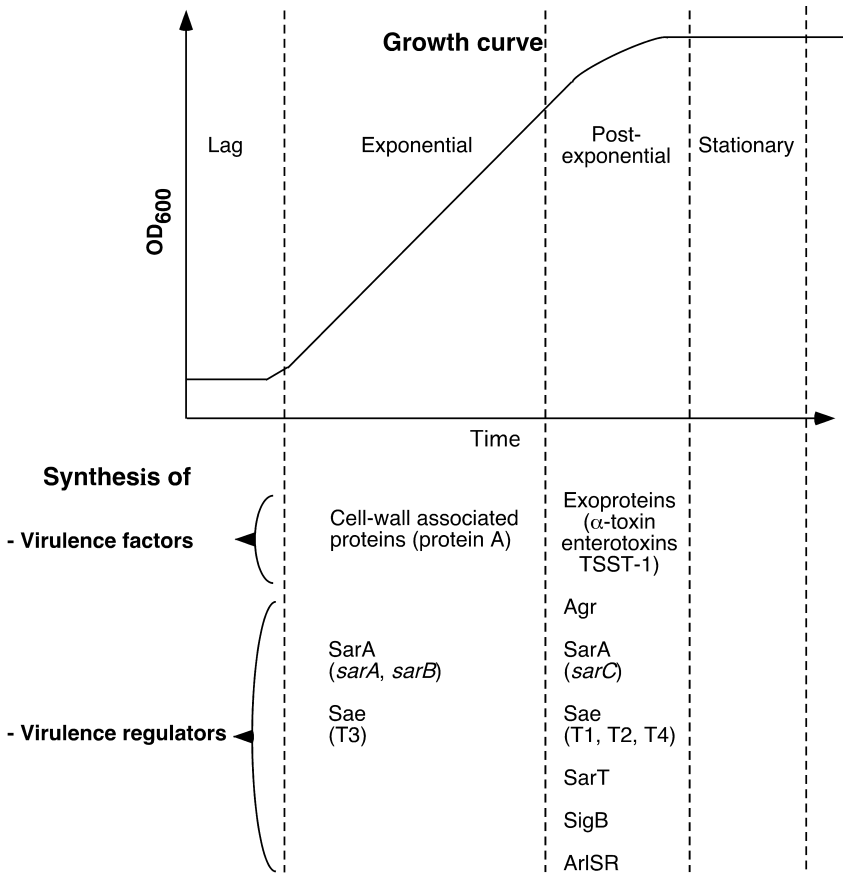



**FIGURE 4.2** Regulation of virulence factors in *S. aureus*. Synthesis of virulence factors and regulators is temporally regulated. A standard *in vitro* growth curve indicates the different growth phases.

the specific peptide modification corresponds to the formation of a cyclic thiolactone bond by a cyclization reaction between an internal cysteine residue and the C terminus of the peptide (Ji *et al.*, 1997). AIP, which is composed of seven to nine residues, is secreted into the extracellular compartment. Maturation and secretion of AIPs are probably provided by the transmembrane protein AgrB (Ji *et al.*, 1995; Zhang *et al.*, 2002). AIP is the ligand for the AgrC sensory component, and RNAIII synthesis is triggered when AIP reaches a threshold concentration that is directly related to cell density (Figures 4.2 and 4.3). This autocrine regulation mechanism indicates that the *agr* locus functions as a quorum-sensing system that detects cell density.

*S. aureus* strains have been classified into four allelic groups (Ji *et al.*, 1997), corresponding to four classes of AIPs, all encoded by the same region of the corresponding *agrD* gene, but distinct in their sequence and/or modification (Mayville *et al.*, 1999). One specific aspect of the Agr system is that AIPs that activate virulence expression in one group of *S. aureus* strains also inhibit the *agr* signaling pathway of all strains with other AIP groups (Lyon *et al.*, 2000).

### *The sarA locus*

A second global regulatory locus, termed the *s*taphylococcal *a*ccessory *r*egulatory (*sarA*) locus, has been described. This locus is also necessary for α-toxin production (Cheung and Ying, 1994). The *sarA* gene encodes the 15-kDa protein SarA transcribed by three overlapping transcripts (Cheung and Projan, 1994). These transcripts (*sarA*, *sarC,* and *sarB*) are initiated from three different promoters, *sarA* at P1, *sar*C at P3, and *sarB* at P2, but are probably terminated at the same stem-loop sequence (Figure 4.3) (Bayer *et al.*, 1996; Manna *et al.*, 1998). P1 and P2 are $\sigma^A$-specific promoters, whereas P3 is recognised by the stress response $\sigma^B$ factor. The SarA protein is produced during all growth phases since *sarA* and *sarB* are synthesised from mid to exponential phase and *sarC* during late-exponential and stationary phase (Manna *et al.*, 1998) (Figure 4.2). SarA, which is a DNA-binding protein, recognizes and binds to a conserved A/T-rich binding site present upstream of the promoter sequence of its target genes (Chien *et al.*, 1999). The SarA protein directly interacts with the *agr* locus by binding to several binding sites present in the *agr-P2-P3* promoter region and up-

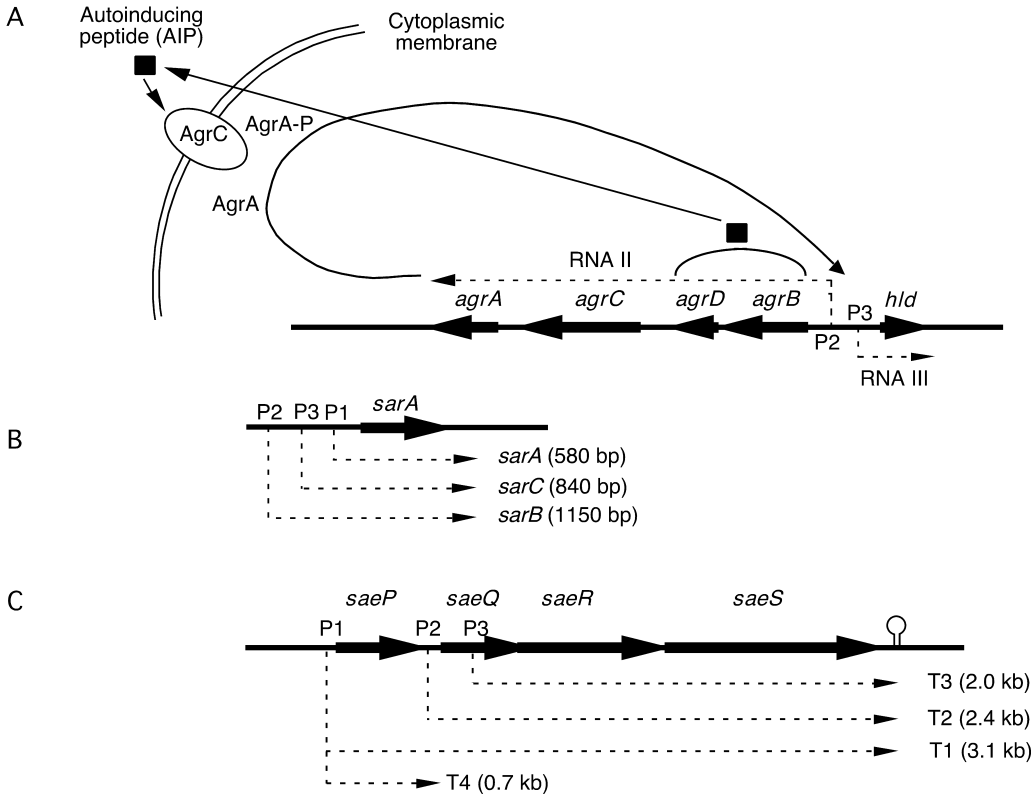



**FIGURE 4.3** Genetic organization of several global regulator genes involved in the regulation of *hla* expression. Dotted lines represent the transcripts. A. The *agr* locus. B. The *sarA* locus. C. The *sae* locus.

regulates the *agr-P2-P3* promoters (Chien *et al.*, 1999; Morfeldt *et al.*, 1996; Rechtin *et al.*, 1999).

### *The Sae system*

The *sae* locus (for *s*taphylococcal *a*ccessory *e*lement) is a four-component system composed of SaeP (or ORF4) and SaeQ (or ORF3), the functions of which are unknown (Novick and Jiang, 2003; Steinhuber *et al.*, 2003), and SaeR and SaeS, which show strong sequence similarity to the response regulators and histidine kinases of two-component systems (Giraudo *et al.*, 1999). These ORFs are transcribed by four overlapping transcripts (T1 to T4) initiated from three promoters (P1 to P3) (Novick and Jiang, 2003; Steinhuber *et al.*, 2003) (Figure 4.3). The T1 transcript initiates at P1 upstream of *saeP*, T2 initiates at P2 upstream of *saeQ*, and T3 initiates at P3 upstream of *saeR*. T1, T2, and T3 mRNAs have a common 3′ end corresponding to a terminator downstream of *saeS*. T4 is a small monocistronic mRNA that includes *saeP* (Steinhuber *et al.*, 2003) (Figure 4.3). The expression of the *sae* genes is controlled by growth phase. T3 is mainly expressed during the exponential phase and disappears when T1, T2, and T4 appear during the post-exponential phase (Figure 4.2). The four transcripts are up-regulated by *agr* and *sarA* (Novick and Jiang, 2003). The Sae system is required for full expression of α- and β-toxin genes (Giraudo *et al.*, 1997; Giraudo *et al.*, 1994) and the action of Sae on *hla* appears to be independent from *agr* and *sarA* (Giraudo *et al.*, 1997).

### *SarA homologues*

Another regulator Rot (*r*epressor *o*f *t*oxin) (15.6 kDa) modifies the expression of toxin genes. Mutation in *rot* partially restores α-toxin production when *agr* is deleted (McNamara *et al.*, 2000). Rot belongs to the Sar family of transcriptional factors of *S. aureus*. Rot activity is down-regulated by the Agr system, whereas the transcription of *rot* is not modified by the Agr system. The mechanism by which the Agr system controls Rot regulation is not known (McNamara *et al.*, 2000).

SarT (16.1 kDa) is another homologue of SarA and is also a DNA-binding protein. The *sarT* transcript is mainly detected during the post-exponential phase (Figuew 4.2) and down-regulates *hla* expression (Schmidt *et al.*, 2001).

Finally, another Sar homologue has an indirect role in the regulatory network of toxin gene expression.

SarU (29 kDa) consists of two distinct domains, each showing sequence similarities with SarA and SarT. Mutation in *sarU* has been shown to attenuate the synthesis of RNAII and RNAIII and consequently the expression of *agr*-target genes such as *hla* (Manna and Cheung, 2003).

### *The Sigma factor B ($\sigma^B$)*

The RNA polymerase core enzyme consists of four subunits ($\alpha^2\beta\beta'$). The interaction between this core enzyme and a σ factor results in an RNA polymerase holoenzyme that can detect specific promoter sequences and activate transcription in various conditions. *S. aureus* has only one of these alternative sigma factors, $\sigma^B$ (Wu *et al.*, 1996). $\sigma^B$ plays a major role in bacterial response to environmental stress and energy depletion. In *S. aureus*, $\sigma^B$ is part of a complex network of regulatory genes controlling the expression of accessory genes. It interacts with other regulatory genes such as transcriptional factors to modulate gene expression, but it is also able to directly regulate the expression of several genes that possess $\sigma^B$-dependent promoters (Kullik *et al.*, 1998).

The *sigB* gene encoding SigB ($\sigma^B$) belongs to a chromosomal regulon of four *S. aureus* genes (*rsbU*, *rsbV*, *rsbW,* and *sigB*) (Wu *et al.*, 1996). Regulation of SigB depends on the phosphorylation state of RsbV. RsbW, an anti-sigma factor, binds to RsbV, an anti-anti-sigma factor, or to SigB depending on the culture conditions encountered (Bronner *et al.*, 2004). In the absence of stress, RsbV is phosphorylated and unable to bind to RsbW. Thus, RsbW binds to SigB, and the resulting RsbW-SigB complexes inactivate SigB. In conditions of stress, RsbV is dephosphorylated and thus able to interact with RsbW, releasing activated SigB (Giachino *et al.*, 2001; Miyazaki *et al.*, 1999). The *sigB* gene is regulated by two different pathways. The first pathway dependent of RsbU is initiated in response to environmental stress such as salt or heat shock. RsbV is dephosphorylated by RsbU and binds to RsbW (Miyazaki *et al.*, 1999). The second pathway independent of RsbU is activated when conditions such as stationary growth phase decrease the intracellular ATP/ADP ratio. In these conditions, RsbV is not phosphorylated and associates with RsbW (Palma and Cheung, 2001). $\sigma^B$ is mainly active during the late exponential phase and its activity decreases upon entry to the stationary phase (Figure 4.2). The expression of *hla* is up-regulated in *sigB*$^-$ mutants (Giachino *et al.*, 2001; Palma and Cheung, 2001; Ziebandt *et al.*, 2001). Furthermore, $\sigma^B$ up-regulates the *sarC* transcript of the *sarA* locus and down-regulates *agr* expression (Bischoff *et al.*, 2001).

### *Other two-component systems*

The ArlSR system (for *a*utolysis-*r*elated *l*ocus) is a two-component system (Fournier *et al.*, 2001). Expression of the *arlRS* locus is growth-phase dependent and peaks in the post-exponential phase. The ArlSR system decreases the production of virulence factors by down-regulating the transcription of some of these genes including α- and β-toxin genes (*hlb*). The Arl system may act on *hla* and *hlb* expression by down-regulating RNAII and RNAIII transcription, consequently decreasing the expression of virulence genes regulated by RNAIII (Fournier *et al.*, 2001).

The SrrAB system (for *s*taphylococcal *r*espiratory *r*esponse) (or SrhSR) is another two-component system (Throup *et al.*, 2001; Yarwood *et al.*, 2001). SrrAB regulates the expression of virulence factors, especially in microaerobic conditions. It also modulates the production of several proteins implicated in energy metabolism The *srrAB* gene is up-regulated in anaerobic conditions. SrrAB regulates the expression of virulence genes such as the TSST-1 gene either by modification of the RNAIII level or by a pathway independent of RNAIII synthesis (Yarwood *et al.*, 2001).

## *In vitro* genetic regulation of toxins

### *Regulation of Cytotoxins*

Most studies have found that α-toxin mRNA levels are directly linked to extracellular toxin levels, indicating that toxin production is mostly regulated at the transcriptional level. The α-toxin is produced at the transition from late exponential to post-exponential phase (Figure 4.2). Regulation of *hla* expression is complex: regulatory elements such as RNAIII and SarA act directly by binding to the promoter region of *hla* (Chien *et al.*, 1999; Novick *et al.*, 1993), whereas others act indirectly. At the beginning of the growth phase, the transcription of *hla* is repressed by Rot and/or SarT (Bronner *et al.*, 2004) (Figure 4.4). The *agr* AIP reaches its threshold around mid-exponential phase, activating *agr* expression and RNAIII synthesis. RNAIII stimulates the expression of *hla*. SarA also induces *hla* and *hlb* expression (Cheung and Ying, 1994), and it is likely that SarA up-regulates *hla* either directly or by down-regulating the *hla* repressor, SarT (Schmidt *et al.*, 2001). Furthermore, during the exponential and post-exponential phases, RNAIII synthesis is induced by SarA and SarU and decreased by ArlSR. The Sae system seems to act independently of the SarA and Agr pathways, whereas SigB modifies *hla* expression by an unknown pathway (possibly Agr) (Figure 4.4).

### *Regulation of superantigens*

The regulation of the TSST-1 and enterotoxin genes is not as well understood as that of the α-toxin gene. The genes encoding these proteins are all expressed at the mid-exponential phase, like the genes encoding most exoproteins (Novick, 2000) (Figure 4.2). The *tst* gene, which encodes TSST-1, is up-regulated mostly by Agr and slightly by SarA (Chan and Foster, 1998; Recsei *et al.*, 1986). The gene encoding enterotoxin A (*sea*) is not regulated by *agr*, unlike *seb*, *sec,* and *sed*, which all need a functional *agr* for full expression (Tremaine *et al.*, 1993; Tseng *et al.*, 2004).

## *In vivo* regulations of exotoxins

Since exotoxins theorically degrade host tissues, erythrocytes, and phagocytic cells, they may be involved in the dissemination and the spread of bacteria to surrounding tissues. Thus, exotoxin gene expression within the host may be affected by micro-environmental factors such as pH and/or host-specific factors such as the presence of fibrinogen, erythrocytes, or leukocytes. In a guinea pig model of device-related infection, only small amounts of RNAIII are synthesized, whereas *hla* expression is high. Furthermore, mutations in *agr* and *sarA* do not modify *hla* expression, whereas mutation in *sae* down-regulates *hla* expression dramatically (Goerke *et al.*, 2001). In subcutaneous chambers implanted in rabbits, expression of secreted virulence factor genes such as *tst* or *hla* is increased, whereas RNAIII expression is considerably decreased (Yarwood *et al.*, 2002). However, in an experimental abscess infection model, depletion of fibrinogen, which participates in the inflammatory response, significantly represses RNAIII expression and *hla* expression (Rothfork *et al.*, 2003). Thus, *S. aureus* within different host niches may use different regulatory pathways to provide exotoxin synthesis.

The expression of *S. aureus* exotoxin genes is thus modulated by a complex regulatory network. The interactions between the different regulators probably play a major role in the pleiotropic regulation of the virulence factors required to adapt to changes in the extracellular environment and to survive within host niches. The role of this regulatory network *in vivo* is still not clear and awaits further study to elucidate how

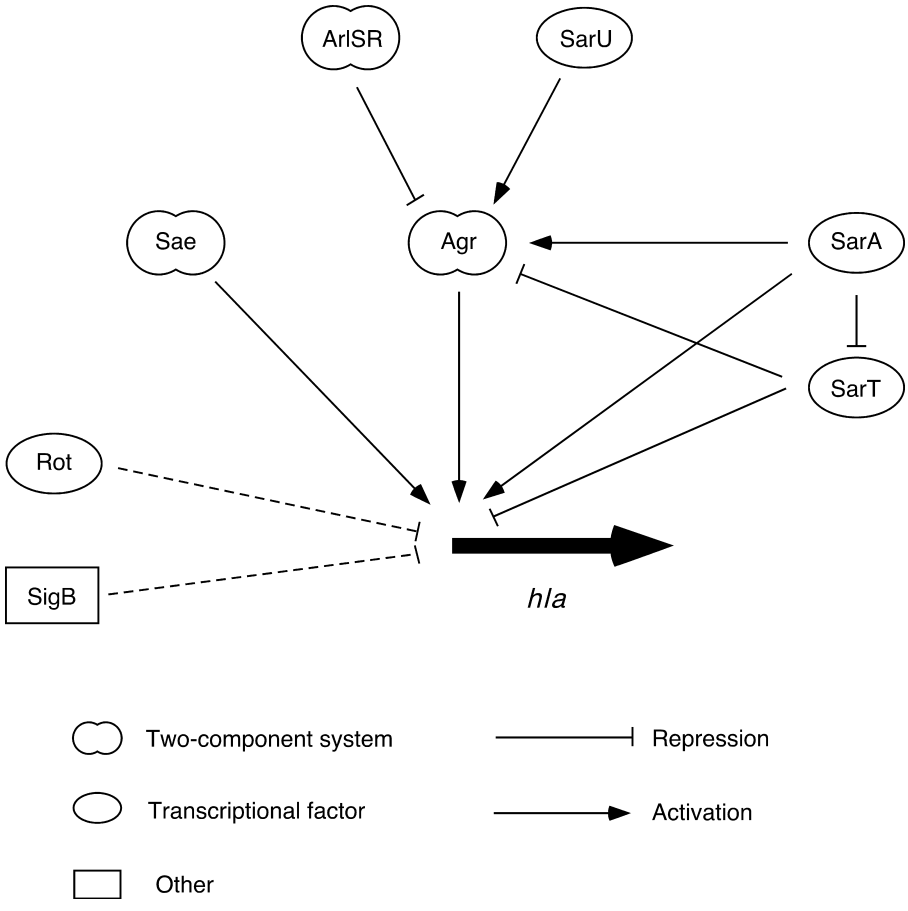



**FIGURE 4.4** Schematic representation of the different pathways regulating *hla* expression. Arrows and perpendicular bars indicate positive and negative regulation, respectively. Dotted lines indicate a possible indirect effect by an unknown pathway. Details of the model are described in the text.

staphylococcal virulence genes are controlled by regulatory factors within the host.

## REFERENCES


Agaisse, H. and Lereclus, D. (1995). How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *J. Bacteriol*. **177,** 6027–6032.

Agaisse, H., Gominet, M., Økstad, O.A., Kolstø, A.B. and Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis. Mol. Microbiol*. **32,** 1043–1053.

Akerley, B.J., Cotter, P.A. and Miller, J.F. (1995). Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell* **80,** 611–620.

Banu, S., Ohtani, K., Yaguchi, H., Swe, T., Cole, S.T., Hayashi, H. and Shimizu, T. (2000). Identification of novel VirR/VirS-regulated genes in *Clostridum perfringens. Mol. Microbiol*. **35,** 854–864.

Ba-Thein, W., Lyristis, M., Ohtani, K., Nisbet, I. T., Hayashi, H., Rood, J. I. and Shimizu, T. (1996) The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens. J. Bacteriol*. **178,** 2514–2520.

Bayer, M.G., Heinrichs, J.H. and Cheung, A.L. (1996). The molecular architecture of the *sar* locus in *Staphylococcus aureus. J. Bacteriol*. **178,** 4563–4570.

Bischoff, M., Entenza, J.M. and Giachino, P. (2001). Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus. J. Bacteriol*. **183,** 5171–5179.

Bock, A. and Gross, R. (2002). The unorthodox histidine kinases BvgS and EvgS are responsive to the oxidation status of a quinone electron carrier. *Eur. J. Biochem*. **269,** 3479–3484.

Bogdan, J.A., Nazario-Larrieu, J., Sarwar, J., Alexander, P. and Blake, M.S. (2001). *Bordetella pertussis* autoregulates pertussis toxin production through the metabolism of cysteine. *Infect. Immun*. **69,** 6823–6830.

Boucher, P.E. and Stibitz, S. (1995). Synergistic binding of RNA polymerase and BvgA phosphate to the pertussis toxin promoter of *Bordetella pertussis. J. Bacteriol*. **177,** 6486–6491.

Boucher, P.E., Maris, A.E., Yang, M.S. and Stibitz, S. (2003). The response regulator BvgA and RNA polymerase alpha subunit C terminal domain bind simultaneously to different faces of the same segment of promoter DNA. *Mol. Cell* **11,** 163–173.

Boucher, P.E., Menozzi, F.D. and Locht, C. (1994). The modular architecture of bacterial response regulators. Insights into the activation mechanism of the BvgA transactivator of *Bordetella pertussis. J. Mol. Biol*. **241,** 363–377.

Boucher, P.E., Yang, M.S., Schmidt, D.M. and Stibitz, S. (2001a). Genetic and biochemical analyses of BvgA interaction with the secondary binding region of the *fha* promoter of *Bordetella pertussis. J. Bacteriol*. **183,** 536–544.

Boucher, P.E., Yang, M.S. and Stibitz, S. (2001b). Mutational analysis of the high-affinity BvgA binding site in the *fha* promoter of *Bordetella pertussis. Mol. Microbiol*. **40,** 991–999.

Brillard, J. and Lereclus, D. (2004). Comparison of cytotoxin *cytK* promoters from *Bacillus cereus* strain ATCC 14579 and from a *B. cereus* food-poisoning strain. *Microbiology* **150,** 2699–2705.

Bronner, S., Monteil, H. and Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol. Rev*. **28,** 183–200.

Callegan, M.C., Kane, S.T., Cochran, D.C., Gilmore, M.S., Gominet, M. and Lereclus, D. (2003). Relationship of PlcR-regulated factors to *Bacillus* endophthalmitis virulence. *Infect. Immun*. **71,** 3116–3124.

Carbonetti, N.H., Romashko, A. and Irish, T.J. (2000). Overexpression of the RNA polymerase alpha subunit reduces transcription of Bvg-activated virulence genes in *Bordetella pertussis. J. Bacteriol*. **182,** 529–531.

Chan, P.F. and Foster, S.J. (1998). Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus. J. Bacteriol*. **180,** 6232–6241.

Cheung, A.L. and Projan, S.J. (1994). Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr. J. Bacteriol*. **176,** 4168–4172.

Cheung, J.K. and Rood, J.I. (2000). The VirR response regulator from *Clostridium perfringens* binds independently to two imperfect direct repeats located upstream of the *pfoA* promoter. *J. Bacteriol*. **182,** 57–66.

Cheung, J. K., Dupuy, B., Deveson, D. S. and Rood, J. I. (2004) The spatial organization of the VirR boxes is critical for VirR-mediated expression of the Perfringolysin O Gene, *pfoA*, from *Clostridium perfringens. J. Bacteriol*. **186,** 3321–3330.

Cheung, A.L. and Ying, P. (1994). Regulation of alpha- and beta-hemolysins by the *sar* locus of *Staphylococcus aureus. J. Bacteriol*. **176,** 580–585.

Chien, Y., Manna, A.C., Projan, S.J. and Cheung, A.L. (1999). SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. *J. Biol. Chem*. **274,** 37169–37176.

Cotter, P.A. and Miller, J.F. (1997). A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol. Microbiol*. **24,** 671–685.

Dupuy, B. and Sonenshein, A.L. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Mol. Microbiol*. **27,** 107–120.

Dupuy, B., Mani, N., Katayama, S. and Sonenshein, A. L. (2005) Transcription activation of a UV-inducible *Clostridium perfringens* bacteriocin gene by a novel sigma factor. *Mol. Microbiol*. **55,** 1196–1206.

Eguchi. Y., Oshima, T., Mori, H., Aono, R., Yamamoto, K., Ishihama, A. and Utsumi, R. (2003). Transcriptional regulation of drug efflux genes by EvgAS, a two-component system in *Escherichia coli. Microbiology* **149,** 2819–2828.

Fedhila, S., Gohar, M., Slamti, L., Nel, P. and Lereclus, D. (2003). The *Bacillus thuringiensis* PlcR-regulated gene *inhA2* is necessary, but not sufficient, for virulence. *J. Bacteriol*. **185,** 2820–2825.

Fedhila, S., Nel, P. and Lereclus, D. (2002). The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. *J. Bacteriol*. **184,** 3296–3304.

Fournier, B., Klier, A. and Rapoport, G. (2001). The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus. Mol. Microbiol*. **41,** 247–261.

Fujihara, H., Walker, L.A., Gong, M.C., Lemichez, E., Boquet, P., Somlyo, A.V. and Somlyo, A.P. (1997). Inhibition of RhoA translocation and calcium sensitization by *in vivo* ADP-ribosylation with the chimeric toxin DC3B. *Mol. Biol. Cell* **8,** 2437–2447.

Gallegos, M.T., Schleif, R., Bairoch, A., Hofmann, K. and Ramos, J.L. (1997). AraC/XylS family of transcriptional regulators. *Microbiol. Mol. Rev*. **61,** 393–410.

Giachino, P., Engelmann, S. and Bischoff, M. (2001). Sigma(B) activity depends on RsbU in *Staphylococcus aureus. J. Bacteriol*. **183,** 1843–1852.

Giraudo, A.T., Calzolari, A., Cataldi, A.A., Bogni, C. and Nagel, R. (1999). The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. *FEMS Microbiol. Lett*. **177,** 15–22.

Giraudo, A.T., Cheung, A.L. and Nagel, R. (1997). The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. *Arch. Microbiol*. **168,** 53–58.

Giraudo, A.T., Raspanti, C.G., Calzolari, A. and Nagel, R. (1994). Characterization of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. *Can. J. Microbiol*. **40,** 677–681.

Goerke, C., Fluckiger, U., Steinhuber, A., Zimmerli, W. and Wolz, C. (2001). Impact of the regulatory loci *agr, sarA*, and *sae* of *Staphylococcus aureus* on the induction of alpha-toxin during device-related infection resolved by direct quantitative transcript analysis. *Mol. Microbiol*. **40,** 1439–1447.

Gohar, M., Økstad, O.A., Gilois, N., Sanchis, V., Kolstø, A.-B. and Lereclus, D. (2002). Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. *Proteomics* **2,** 784–791.

Gominet, M., Slamti, L., Gilois, N., Rose, M. and Lereclus, D. (2001). Oligopeptide permease is required for expression of the *Bacillus thuringiensis* PlcR regulon and for virulence. *Mol. Microbiol*. **40,** 963–975.

Goyard, S., Bellalou, J., Mireau, H. and Ullmann, A. (1994). Mutations in the *Bordetella pertussis bvgS* gene that confer altered expression of the *fhaB* gene in *Escherichia coli. J. Bacteriol*. **176,** 5163–5166.

Helmann, J.D. (2002). The extracytoplasmic function (ECF) sigma factors. *Adv. Microbial. Physiol*. **46,** 47–110.

Hirst, T. R. (1999). Cholera toxin and *Escherichia coli* heat-labile enterotoxin. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer) Academic Press, London, pp. 104–129.

Hot, D., Antoine, R., Renauld-Mongenie, G., Caro, V., Hennuy, B., Levillain, E., Huot, L., Wittmann, G., Poncet, D., Jacob-Dubuisson, F., Guyard, C., Rimlinger, F., Aujame, L., Godfroid, E., Guiso, N., Quentin-Millet, M.J., Lemoine, Y. and Locht, C. (2003). Differential modulation of *Bordetella pertussis* virulence genes as evidenced by DNA microarray analysis. *Mol. Genet. Genomics* **269,** 475–486.

Huang, I.H., Waters, M., Grau, R.R. and Sarker, M.R. (2004). Disruption of the genes (*spoOA*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiol. Lett*. **233,** 233–240.

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M. and von Eichel-Streiber, C. (1996). Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile. Eur. J. Biochem*. **244,** 735–742.

Irie, Y., Mattoo, S. and Yuk, M.H. (2004). The Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica. J. Bacteriol*. **186,** 5692–5698.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M., Ehrlich, S.D., Overbeek, R. and Kyrpides, N. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis. Nature* **423,** 87–91.

Ji, G., Beavis, R. and Novick, R.P. (1997). Bacterial interference caused by autoinducing peptide variants. *Science* **276,** 2027–2030.

Ji, G., Beavis, R.C. and Novick, R.P. (1995). Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. USA* **92,** 12055–12059.

Johnson, E. A. (2000) In: *Gram Positive Pathogens* (eds. V.A. Fischetti, J.J. Ferretti, D.A. Portnoy, R.P. Novick and J.I. Rood) *Neurotoxigenic Clostridia*, pp. 540–550, ASM Press, Washington.

Kaji, M., Matsushita, O., Tamai, E., Miyata, S., Taniguchi, Y., Shimamoto, S., Katayama, S., Morita, S. and Okabe, A. (2003). A novel type of DNA curvature present in a *Clostridium perfringens* ferredoxin gene: characterization and role in gene expression. *Microbiology* **149,** 3083–3091.

Karimova, G. and Ullmann, A. (1997). Characterization of DNA binding sites for the BvgA protein of *Bordetella pertussis. J. Bacteriol*. **179,** 3790–3792.

Karlsson, S., Burman, L.G. and Akerlund, T. (1999). Suppression of toxin production in *Clostridium difficile* VPI10463 by amino acids. *Microbiology* **145,** 1683–1693.

Karlsson, S., Dupuy, B., Mukherjee, K., Norin, E., Burman, L.G. and Akerlund, T. (2003). Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. *Infect. Immun*. **71,** 1784–1793.

Katayama, S., Matsushita, O., Jung, C.M., Minami, J. and Okabe, A. (1999). Promoter upstream bent DNA activates the transcription of the *Clostridium perfringens* phospholipase C gene in a low temperature-dependent manner. *EMBO J.* **18,** 3442–3450.

Katayama, S., Matsushita, O., Tamai, E., Miyata, S. and Okabe, A. (2001). Phased A-tracts bind to the alpha subunit of RNA polymerase with increased affinity at low temperature. *FEBS Letters* **509,** 235–238.

Kinnear, S.M., Marques, R.R. and Carbonetti, N.H. (2001). Differential regulation of Bvg-activated virulence factors plays a role in *Bordetella pertussis* pathogenicity. *Infect. Immun*. **69,** 1983–1993.

Koehler, T.M. (2002). *Bacillus anthracis* genetics and virulence gene regulation. *Curr. Top. Microbiol. Immunol*. **271,** 143–164.

Kolstø, A.B., Lereclus, D. and Mock, M. (2002). Genome structure and evolution of the *Bacillus cereus* group. *Curr. Top. Microbiol. Immunol*. **264,** 95–108.

Kullik, I., Giachino, P. and Fuchs, T. (1998). Deletion of the alternative sigma factor sigmaB in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. *J. Bacteriol*. **180,** 4814–4820.

Lacey, B.W. (1960). Antigenic modulation of *Bordetella pertussis. J. Hyg*. (Lond). **58,** 57–93.

Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiessner-Gunkel, E., Stewart, G.S. and Scherer, S. (1998). *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. *Int. J. Syst. Bacteriol*. **48,** 1373–1382.

Leppla, S.H. (2000). Anthrax toxin. In: *Bacterial Protein Toxins* (eds. K. Aktories and I. Just), pp. 445–472, Springer, Berlin.

Lereclus, D., Agaisse, H., Gominet, M., Salamitou, S. and Sanchis, V. (1996). Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. *J. Bacteriol*. **178,** 2749–2756.

Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S. and Gominet, M. (2000). Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. *Int. J. Med. Microbiol.* **290,** 295–299.

Leslie, P.H. and Gardner, A.D. (1931). The phases of *Haemophilus pertussis. J. Hyg*. **31,** 423–434.

Lina, G., Jarraud, S., Ji, G., Greenland, T., Pedraza, A., Etienne, J., Novick, R.P. and Vandenesch, F. (1998). Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus. Mol. Microbiol*. **28,** 655–662.

Lund, T., DeBuyser, M.-L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. **38,** 254–261.

Lyon, G.J., Mayville, P., Muir, T.W. and Novick, R.P. (2000). Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, *AgrC. Proc. Natl. Acad. Sci. USA* **97,** 13330–13335.

Manetti, R., Arico, B., Rappuoli, R. and Scarlato, V. (1994). Mutations in the linker region of BvgS abolish response to environmental

signals for the regulation of the virulence factors in *Bordetella pertussis. Gene* **150,** 123–127.

Mani, N. and Dupuy, B. (2001). Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. *Proc. Natl. Acad. Sci. USA* **98,** 5844–5849.

Mani, N., Lyras, D., Barroso, L., Howarth, P., Wilkins, T., Rood, J.I., Sonenshein, A.L. and Dupuy, B. (2002). Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. *J. Bacteriol*. **184,** 5971–5978.

Manna, A.C., Bayer, M.G. and Cheung, A.L. (1998). Transcriptional analysis of different promoters in the *sar* locus in *Staphylococcus aureus. J. Bacteriol*. **180,** 3828–3836.

Manna, A.C. and Cheung, A.L. (2003). *sarU*, a *sarA* homolog, is repressed by SarT and regulates virulence genes in *Staphylococcus aureus. Infect. Immun*. **71,** 343–353.

Marques, R.R. and Carbonetti, N.H. (1997). Genetic analysis of pertussis toxin promoter activation in *Bordetella pertussis. Mol. Microbiol*. **24,** 1215–1224.

Martinez de Tejada, G., Cotter, P.A., Heininger, U., Camilli, A., Akerley, B.J., Mekalanos, J.J. and Miller J.F. (1998). Neither the Bvg-phase nor the *vrg6* locus of *Bordetella pertussis* is required for respiratory infection in mice. *Infect. Immun*. **66,** 2762–2768.

Martinez de Tejada, G, Miller, J.F. and Cotter, P.A. (1996). Comparative analysis of the virulence control systems of *Bordetella pertussis* and *Bordetella bronchiseptica. Mol. Microbiol*. **22,** 895–908.

Marvaud, J.C., Eisel, U., Binz, T., Niemann, H. and Popoff, M.R. (1998a). *tetR* is a positive regulator of the Tetanus toxin gene in *Clostridium tetani* and is homologous to *botR. Infect. Immun*. **66,** 5698–5702.

Marvaud, J.C., Gibert, M., Inoue, K., Fujinaga, V., Oguma, K. and Popoff, M.R. (1998b). *bot*R is a positive regulator of botulinum neurotoxin and associated non toxic protein genes in *Clostridium botulinum A. Mol. Microbiol*. **29,** 1009–1018.

Matsushita, C., Matsushita, O., Katayama, S., Minami, J., Takai, K. and Okabe, A. (1996) An upstream activating sequence containing curved DNA involved in activation of the *Clostridium perfringens plc* promoter. *Microbiology* **142,** 2561–2566.

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R.P. and Muir, T.W. (1999). Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. *Proc. Natl. Acad. Sci. USA* **96,** 1218–1223.

McCormick, J.K., Yarwood, J.M. and Schlievert, P.M. (2001). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55,** 77–104.

McGowan, S., Lucet, I. S., Cheung, J. K., Awad, M. M., Whisstock, J. C. and Rood, J. I. (2002) The FxRxHrS motif: A conserved region essential for DNA binding of the VirR response regulator from *Clostridium perfringens. J. Mol. Biol*. **322,** 997–1011.

McGowan, S., O'Connor, J. R., Cheung, J. K. and Rood, J. I. (2003). The SKHR motif is required for biological function of the VirR response regulator from *Clostridium perfringens. J. Bacteriol*. **185,** 6205–6208.

McNamara, P.J., Milligan-Monroe, K.C., Khalili, S. and Proctor, R.A. (2000). Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus. J. Bacteriol*. **182,** 3197–3203.

Merkel, T.J., Barros, C. and Stibitz, S. (1998a). Characterization of the *bvgR* locus of *Bordetella pertussis. J. Bacteriol*. **180,** 1682–1690.

Merkel, T.J., Stibitz, S., Keith, J.M., Leef, M. and Shahin, R. (1998b). Contribution of regulation by the *bvg* locus to respiratory infection of mice by *Bordetella pertussis. Infect. Immun*. **66,** 4367–4373.

Mignot, T., Mock, M., Robichon, D., Landier, A., Lereclus, D. and Fouet, A. (2001). The incompatibility between the PlcR- and AtxA-controlled regulons may have selected a nonsense mutation in *Bacillus anthracis. Mol. Microbiol*. **42,** 1189–1198.

Miller, M.B. and Bassler, B.L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol*. **55,** 165–199.

Miyazaki, E., Chen, J.M., Ko, C. and Bishai, W.R. (1999). The *Staphylococcus aureus rsbW* (*orf159*) gene encodes an anti-sigma factor of SigB. *J. Bacteriol*. **181,** 2846–2851.

Mock, M. and Fouet, A. (2001). Anthrax. *Annu. Rev. Microbiol*. **55,** 647–671.

Monack, D.M., Arico, B., Rappuoli, R. and Falkow S. (1989). Phase variants of *Bordetella bronchiseptica* arise by spontaneous deletions in the *vir* locus. *Mol. Microbiol*. **3,** 1719–1728.

Moncrief, J.S., Barroso, L.A. and Wilkins, T.D. (1997). Positive regulation of *Clostridium difficile* toxins. *Infect. Immun*. **65,** 1105–1108.

Morfeldt, E., Taylor, D., von Gabain, A. and Arvidson, S. (1995). Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *EMBO J*. **14,** 4569–4577.

Morfeldt, E., Tegmark, K. and Arvidson, S. (1996). Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus. Mol. Microbiol*. **21,** 1227–1237.

Nakamura, L.K. (1994). DNA relatedness among *Bacillus thuringiensis* serovars. *Int. J. Syst. Bacteriol*. **44,** 125–129.

Nakano, M.M., Zuber, P. and Sonenshein, A.L. (2004). Toxin gene regulation in *Clostridium*. In: *Strict and facultative anaerobes. Medical and environmental aspects*. (eds. Nakano, M.M. and Zuber, P.), pp. 199–209. Horizon Bioscience, Wymondham, UK.

Novick, R.P. (2000). Pathogenicity factors and their regulation. In "Gram-positive pathogens" (Ed V. A. Fischetti and R. P. Novick and J. J. Ferreti and D. A. Portnoy and J. I. Rood), pp. 392–407. American Society for Microbiology, Washington, D.C.

Novick, R.P. and Jiang, D. (2003). The staphylococcal *saeRS* system coordinates environmental signals with *agr* quorum sensing. *Microbiology* **149,** 2709–2717.

Novick, R.P., Projan, S.J., Kornblum, J., Ross, H.F., Ji, G., Kreiswirth, B., Vandenesch, F. and Moghazeh, S. (1995). The *agr P2* operon: an autocatalytic sensory transduction system in *Staphylococcus aureus. Mol. Gen. Genet*. **248,** 446–458.

Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B. and Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J*. **12,** 3967–3975.

Ohtani, K., Kawsar, H.I., Okumura, K., Hayashi, H. and Shimizu, T. (2003). The VirR/VirS regulatory cascade affects transcription of plasmid-encoded putative virulence genes in *Clostridium perfringens* strain 13. *FEMS Microbiol. Lett*. **222,** 137–141.

Økstad, O.A., Gominet, M., Purnelle, B., Rose, M., Lereclus, D. and Kolstø, A.-B. (1999). Sequence analysis of three *Bacillus cereus* loci under PlcR virulence gene regulator control. *Microbiology* **145,** 3129–3138.

Palma, M. and Cheung, A.L. (2001). Sigma(B) activity in *Staphylococcus aureus* is controlled by RsbU and an additional factor(s) during bacterial growth. *Infect. Immun*. **69,** 7858–7865.

Parkhill, J., Sebaihia, M., Preston, A., Murphy, L.D., Thomson, N., Harris, D.E., Holden, M.T., Churcher, C.M., Bentley, S.D., Mungall, K.L., Cerdeno-Tarraga, A.M., Temple, L., James, K., Harris, B., Quail, M.A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A., Davis, P., Doggett, J., Feltwell, T., Goble, A., Hamlin, N., Hauser, H., Holroyd, S., Jagels, K., Leather, S., Moule, S., Norberczak, H., O'Neil, S., Ormond, D., Price, C., Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B.G. and Maskell, D.J. (2003). Comparative analysis of the genome sequences of *Bordetella per-*

*tussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica. Nat. Genet*. **35,** 32–40.

Payne, S.M. (2003). Regulation of bacterial toxin synthesis by iron. In: Bacterial Protein Toxins, eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski, and R. Rappuoli, Washington, D.C.: ASM Press, 25–38.

Passerini de Rossi, B.N., Friedman, L.E., Belzoni, C.B., Savino, S., Arico, B., Rappuoli, R., Masignani, V. and Franco, M.A. (2003). Vir90, a virulence-activated gene coding for a *Bordetella pertussis* iron-regulated outer membrane protein. *Res. Microbiol*. **154,** 443–450.

Perego, M. and Hoch, J.A. (2002). Two component systems, phosphorelays, and regulation of their activities by phosphatases. In: *Bacillus subtilis* and its closest relatives, eds. A.L. Sonenshein, J.A. Hoch, and R. Losick, Washington, D. C.: ASM Press, 473–481.

Perraud, A.L., Kimmel, B., Weiss, V. and Gross, R. (1998). Specificity of the BvgAS and EvgAS phosphorelay is mediated by the C-terminal HPt domains of the sensor proteins. *Mol. Microbiol*. **27,** 875–887.

Perraud, A.L., Rippe, K., Bantscheff, M., Glocker, M., Lucassen, M., Jung, K., Sebald, W., Weiss, V. and Gross, R. (2000). Dimerization of signaling modules of the EvgAS and BvgAS phosphorelay systems. *Biochim. Biophys. Acta* **1478,** 341–354.

Pomerantsev, A.P., Kalnin, K.V., Osorio, M. and Leppla, S.H. (2003). Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. *Infect. Immun*. **71,** 6591–6606.

Pomerantsev, A.P., Pomerantseva, O.M. and Leppla, S.H. (2004). A spontaneous translational fusion of *Bacillus cereus* PlcR and PapR activates transcription of PlcR-dependent genes in *Bacillus anthracis* via binding with a specific palindromic sequence. *Infect. Immun*. **72,** 2814–2823.

Preston, A., Maxim, E., Toland, E., Pishko, E.J., Harvill, E.T., Caroff, M. and Maskell, D.J. (2003). *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. *Mol. Microbiol*. **48,** 725–736.

Priest, F.G., Kaji, D.A., Rosato, Y.B. and Canhos, V.P. (1994). Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. *Microbiology* **140,** 1015–1022.

Projan, S.J. and Novick, R.P. (1997). The molecular basis of pathogenicity. In "The staphylococci in human disease" (Ed K. B. Crossley and G. L. Archer), pp. 55–81. Churchill Livingstone, New-York, N.Y.

Quinn, C.P. and Minton, N.P. (2001). Clostridial neurotoxins. In: *Clostridia*. (eds. Bahl, H. and Dürre, P.) pp. 211–250 Wiley-VCH: Veinheim.

Raffestin, S., Marvaud, J.C., Cerrato, R., Dupuy, B. and Popoff, M.R. (2004). Organization and regulation of the neurotoxin genes in *Clostridium botulinum* and *Clostridium tetani. Anaerobe* **10,** 93–100.

Rasko, D.A., Ravel, J., Økstad, O.A., Helgason, E., Cer, R.Z., Jiang, L., Shores, K.A., Fouts, D.E., Tourasse, N.J., Angiuoli, S.V., Kolonay, J., Nelson, W.C., Kolstø, A.-B., Fraser, C.M. and Read, T.D. (2004). The genome sequence of *Bacillus cereus* ATCC 10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic Acids Res*. **32,** 977–988.

Rechtin, T.M., Gillaspy, A.F., Schumacher, M.A., Brennan, R.G., Smeltzer, M.S. and Hurlburt, B.K. (1999). Characterization of the SarA virulence gene regulator of *Staphylococcus aureus. Mol. Microbiol*. **33,** 307–316.

Recsei, P., Kreiswirth, B., O'Reilly, M., Schlievert, P., Gruss, A. and Novick, R.P. (1986). Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr. Mol. Gen. Genet*. **202,** 58–61.

Rood, J.I. (1998). Virulence genes of *Clostridium perfringens. Annu. Rev. Microbiol*. **52,** 333–360.

Rothfork, J.M., Dessus-Babus, S., Van Wamel, W.J., Cheung, A.L. and Gresham, H.D. (2003). Fibrinogen depletion attenuates *Staphyloccocus aureus* infection by preventing density-dependent virulence gene up-regulation. *J. Immunol*. **171,** 5389–5395.

Rupnik, M., Dupuy, B., Fairweather, N. F., Gerding, D. N., Johnson, S., Just, I., Lyerly, D. M., Popoff, M. R., Rood, J. I., Sonenshein, A. L., Thelestam, M., Wren, B. W., Wilkins, T. D. and Von Eichel-Streiber, C. (2005) Revised nomenclature of *Clostridium difficile* toxins and associated genes. *J. Med. Microbiol*. **54,** 113–117.

Salamitou, S., Ramisse, F., Brehélin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. and Lereclus, D. (2000). The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology* **146,** 2825–2832.

Schmidt, J.J., Stafford, R.G. and Millard, C.B. (2001). High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. *Anal. Biochem*. **296,** 130–137.

Schmidt, K.A., Manna, A.C., Gill, S. and Cheung, A.L. (2001). SarT, a repressor of alpha-hemolysin in *Staphylococcus aureus. Infect. Immun*. **69,** 4749–4758.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev*. **62,** 775–806.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. and Hayashi, H. (2002a). Complete genome sequence of *Clostridium perfringens,* an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* **99,** 996–1001.

Shimizu, T., Shima, K., Yoshino, K., Yonesawa, K., Shimizu, T. and Hayashi, H. (2002b). Proteome and transcriptome analysis of the virulence genes regulates by the VirR/VirS system in *Clostridium perfringens. J. Bacteriol*. **184,** 2587–2594.

Shimizu, T., Yaguchi, H., Ohtani, K., Banu, S. and Hayashi, H. (2002c). Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. *Mol. Microbiol*. **43,** 257–265.

Slamti, L. and Lereclus, D. (2002). A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. *EMBO J*. **21,** 4550–4559.

Slamti, L. and Lereclus, D. (2005). Specificity and polymorphism of the PlcR-PapR quorum-sensing system in the *Bacillus cereus* group. *J. Bacteriol*. (in press)

Slamti, L., Perchat, S., Gominet, M., Vilas-Bôas, G., Fouet, A., Mock, M., Sanchis, V., Chaufaux, J., Gohar, M. and Lereclus, D. (2004). Distinct mutations in PlcR explain why some strains of the *Bacillus cereus* group are nonhemolytic. *J. Bacteriol*. **186,** 3531–3538.

Sonenshein, A.L., Hoch, J.A. and Losick, R. (1993). *Bacillus subtilis* and other Gram-positive bacteria. ASM Press: Washington, D. C.

Sonenshein, A.L., Hoch, J.A. and Losick, R. (2002). *Bacillus subtilis* and its closest relatives. Washington, D. C.: ASM Press.

Steinhuber, A., Goerke, C., Bayer, M.G., Doring, G. and Wolz, C. (2003). Molecular architecture of the regulatory Locus *sae* of *Staphylococcus aureus* and its impact on expression of virulence factors. *J. Bacteriol*. **185,** 6278–6286.

Stibitz, S. (1998). Mutations affecting the alpha subunit of *Bordetella pertussis* RNA polymerase suppress growth inhibition conferred by short C-terminal deletions of the response regulator BvgA. *J. Bacteriol*. **180,** 2484–2492.

Stibitz, S. (2003). Two-component systems. In: Bacterial Protein Toxins, eds. D.L. Burns, J.T. Barbieri, B.H. Iglewski, and R. Rappuoli, Washington , D. C.: ASM Press, 3–23.

Stibitz, S. and Yang, M.S. (1991). Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. *J. Bacteriol*. **173,** 4288–4296.

Stibitz, S., Aaronson, W., Monack, D. and Falkow, S. (1989). Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. *Nature* **338,** 266–269.

Stock, J.B., Ninfa, A.J. and Stock, A.M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev*. **53,** 450–490.

Titball, R. W. and Rood, J. I. (2002) In "Molecular Medical Microbiology"(ed M. Sussman) *Clostridium perfringens*: Wound Infections., pp. 1875–1903, Academic Press, London,

Throup, J.P., Zappacosta, F., Lunsford, R.D., Annan, R.S., Carr, S.A., Lonsdale, J.T., Bryant, A.P., McDevitt, D., Rosenberg, M. and Burnham, M.K. (2001). The *srhSR* gene pair from *Staphylococcus aureus*: genomic and proteomic approaches to the identification and characterization of gene function. *Biochemistry* **40,** 10392–10401.

Toyonaga, T., Matsushita, O., Katayama, S.-I., Minami, J. and Okabe, A. (1992) Role of the upstream region containing an intrinsic DNA curvature in the negative regulation of the phospholipase C gene of *Clostridium perfringens*. *Microbiology Immunol*. **36,** 603–613.

Tremaine, M.T., Brockman, D.K. and Betley, M.J. (1993). Staphylococcal enterotoxin A gene (*sea*) expression is not affected by the accessory gene regulator (*agr*). *Infect. Immun*. **61,** 356–359.

Tseng, C.W., Zhang, S. and Stewart, G.C. (2004). Accessory gene regulator control of staphyloccoccal enterotoxin D gene expression. *J. Bacteriol*. **186,** 1793–1801.

Uhl, M.A. and Miller, J.F. (1994). Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade. *Proc. Natl. Acad. Sci. USA* **91,** 1163–1167.

Wu, S., de Lencastre, H. and Tomasz, A. (1996). Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. *J. Bacteriol*. **178,** 6036–6042.

Yarwood, J.M., McCormick, J.K., Paustian, M.L., Kapur, V. and Schlievert, P.M. (2002). Repression of the *Staphylococcus aureus* accessory gene regulator in serum and *in vivo*. *J. Bacteriol*. **184,** 1095–1101.

Yarwood, J.M., McCormick, J.K. and Schlievert, P.M. (2001). Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. *J. Bacteriol*. **183,** 1113–1123.

Zhang, L., Gray, L., Novick, R.P. and Ji, G. (2002). Transmembrane topology of AgrB, the protein involved in the post-translational modification of AgrD in *Staphylococcus aureus*. *J. Biol. Chem*. **277,** 34736–34742.

Ziebandt, A.K., Weber, H., Rudolph, J., Schmid, R., Hoper, D., Engelmann, S. and Hecker, M. (2001). Extracellular proteins of *Staphylococcus aureus* and the role of SarA and sigma B. *Proteomics* **1,** 480–493.

CHAPTER

# 5

# Toxin secretion systems

*Maria Scott and Maria Sandkvist*

## INTRODUCTION

Toxins and other effector molecules enhance the virulence characteristics of bacteria and play a pivotal role in host disease processes. These proteins must be transported outside of the cell if bacteria are to garner any infective or survival advantage of their production. Bacteria may release these factors into the extracellular milieu, inject the secreted proteins directly into the host cells or attach these proteinaceous substances to their outer surface. Proteins exported by Gram-positive bacteria have only one lipid-bilayer to transverse so it is generally believed that secretion in these organisms is rather simplistic in comparison to Gram-negative organisms. This is because secreted proteins produced by Gram-negative organisms must cross two barriers, the cytoplasmic (inner) and outer membranes. Despite that, both classes of organisms utilize signal-sequence dependent Sec and Tat pathways for export of proteins across the cytoplasmic membrane. Consequently, Gram-negative bacterial evolution diverged to provide complex and highly specialized systems for secretion of bacterial proteins across the outer membrane.

Five extracellular secretion systems produced by Gram-negative organisms have been described to date and they are designated as types I-V. Two of these pathways shuttle proteins that arrive in the periplasmic compartment via the Sec or Tat pathways across the outer membrane. Type V system, consisting of the autotransporter proteins, appears by comparison to be the least complicated. However the autotransporter proteins represent the largest group and may actually exemplify the most flexible system of all secretion pathways since autotransporter proteins, following export across the cytoplasmic membrane, transverse the outer membrane largely without accessory proteins. The type II system transports fully folded proteins across the outer membrane. This system is much more complex and consists of 12-16 proteins that comprise a macromolecular structure that is thought to span both the inner and outer membranes. The type IV secretion system is also complex, consisting of up to 11 proteins, and is thought to be ancestrally related to the type II system yet is distinctly different. Case in point, the type IV system transports ssDNA as well as proteins across the cell envelope. Proteins secreted via the type I and type III pathways completely bypass the periplasmic compartment. They do not rely on Sec or Tat for cytoplasmic membrane transport; they transverse the cell envelope in a single step. In the type I system proteins cross the two membrane barriers through a channel formed by one of three accessory proteins. The type III system, made of approximately 25 components, is one of the most intriguing systems and is commonly called the molecular syringe because proteins secreted via this pathway are injected directly from the cytosol of the bacterial cell into the host cell without encountering the extracellular environment.

In the next few sections important features of the protein secretion pathways known to play a key role in secretion of housekeeping proteins as well as virulence factors will be presented. Salient facts about export of proteins across the cytoplasmic membrane by way of the Sec machinery and twin arginine translocation (Tat) pathway will be described. Also the type V and II secretion systems will be discussed, followed by the type IV system, ending with an overview of types I and III pathways.

## INNER MEMBRANE TRANSLOCATION

### Sec-mediated export of proteins across the cytoplasmic membrane

Proteins emerging from ribosomes must be sorted, sometimes before synthesis is complete, for insertion into the cytoplasmic membrane, delivery to the periplasmic compartment or retention in the cytoplasm. The Sec protein export pathway has an essential role in the sorting and biogenesis of secreted proteins that cross or insert into the cytoplasmic membrane. Nascent polypeptides carry a typical signal-sequence at the amino-terminal end that targets them to the Sec translocon (Dalbey and Kuhn, 2000). The signal sequence is about 20 amino acids long with a short stretch of flanking positively charged residues followed by a hydrophobic region and a consensus proteolytic cleavage site (Dalbey and Kuhn, 2000). Both Gram-negative and Gram-positive organisms utilize the Sec system as the first step in secretion of many proteinaceous factors. The protein-conducting channel and the accessory proteins have been studied extensively in both *Escherichia coli* and *Bacillus subtilis* and comparative analyses reveal a highly conserved pathway. In fact, both eukaryotes and archaea carry homologues of the Sec translocon.

The Sec machinery consists of several components that coordinate export of precursor proteins (see Figure 5.1). First, as the signal sequence of the precursor protrudes from the ribosome it is recognized by the holdase chaperone, SecB, which binds in close proximity to the signal sequence slowing polypeptide rate of folding (Kumamoto and Beckwith, 1983) (Collier *et al.*, 1988) (Hartl *et al.*, 1990). Next, transient but high affinity molecular interactions between SecB and SecA bring the precursor in contact with SecA (Fekkes *et al.*, 1997). The SecA component recognizes the signal sequence of the nascent protein and prevents its folding (Oliver and Beckwith, 1981) (Hunt *et al.*, 2002). Translocation of the nascent protein is initiated following contact of SecA with the SecYEG complex, which causes a dramatic conformational change in the SecA protein. The subsequent binding of ATP drives SecA into the lipid bilayer (Wang *et al.*, 2004). Continued

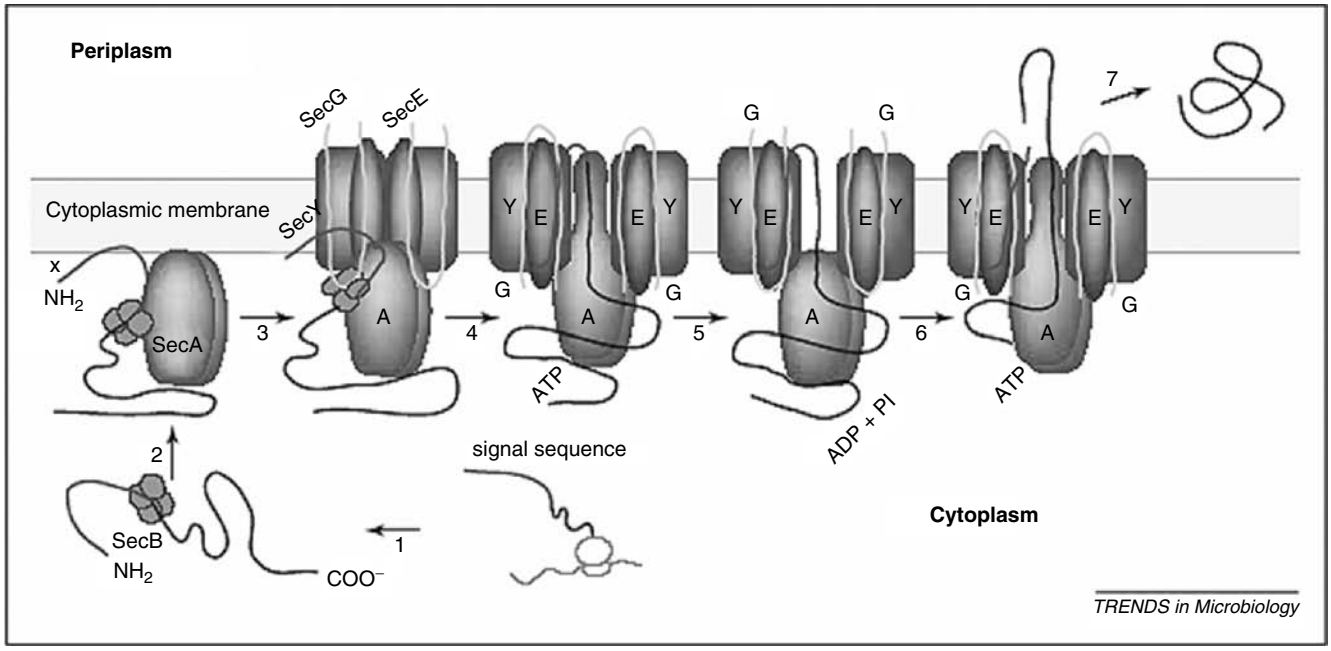



**FIGURE 5.1** Diagram of the Sec translocation process. The nascent polypeptide is represented by the black line. Step 1-3, targeting: The signal sequence of the polypeptide protrudes from the ribosome and is recognized by the SecB chaperone which binds close to the target sequence slowing the rate of polypeptide folding. Transient but high affinity intermolecular interactions between SecB and SecA bring the precursor protein to the translocase. SecA bound to the nascent polypeptide binds to the membrane and docks with the SecYEG heterotrimeric complex. Steps 4 and 5, initiation: The initiation step follows ATP binding to the SecA component resulting in a dramatic conformation change in the SecA protein driving it into the lipid bilayer. Step 6 consists of continued cycles of ATP hydrolysis by SecA in conjunction with the proton motive force to energize unidirectional translocation of the nascent protein across the membrane. Step7, termination occurs on the periplasmic side followed by cleavage of the signal peptide, folding and release of the substrate to the periplasmic space. "Reprinted from *Trends in Microbiology, Vol. 9(10)*, Hiroyuki Mori and Koreaki Ito, The Sec protein-translocation pathway, 494-500©2001, with permission from Elsevier Science Ltd.

cycles of ATP hydrolysis by SecA in conjunction with the proton motive force, energizes unidirectional translocation of the nascent protein across the membrane (Vrontou and Economou, 2004). Steps leading to termination of the process are not clearly defined. However accessory proteins, SecDF-YajC, have been suggested to interact with SecYEG and stabilize transfer of the protein to the periplasmic side of the inner membrane (Duong and Wickner, 1997). When the preprotein emerges on the other side of the membrane the signal sequence is cleaved off by the signal peptidase and folding of the mature protein occurs (Schiebel *et al.*, 1991) (Dalbey *et al.*, 1997).

### *The* E. coli *Sec translocase*

The prevailing model is that the *E. coli* Sec translocon consists of the heterotrimeric complex SecYEG. SecY is an essential component of the translocon in that it forms the protein translocation pore. It is a 48 kDa integral membrane protein that spans the cytoplasmic membrane ten times (Clemons, Jr. *et al.*, 2004). Another principal factor of the translocon is SecE, a 14 kDa inner membrane protein. Mutational analyses and crosslinking studies indicate that SecY and SecE form a stable complex through several points of contact. Equimolar concentrations of SecE and SecY are maintained within the cell (Veenendaal *et al.*, 2001) and any excess SecY is rapidly degraded by membrane bound FtsH (Kihara *et al.*, 1995). SecG (11.5 kDa) spans the membrane twice and also contains a somewhat hydrophobic cytosolic segment (Nishiyama *et al.*, 1996). In addition SecG has the unique capacity to flip-flop in the membrane reversing its topology during membrane insertion of SecA. Thus, SecG is thought to augment SecA ATP binding cycles and play an important role in the SecA membrane insertion-deinsertion process (Nishiyama *et al.*, 1996).

Recently the crystal structure of the Secαγβ complex of *Methanococus jannaschii* was solved to a resolution of 3.2 Å (van den *et al.*, 2004). The archaeal Secαγβ complex corresponds to SecYEG, where Secα and Secγ have exact counterparts in bacterial SecY and SecE; respectively. The Seca and SecG, on the other hand, are not homologues, but may perform similar functions. The archaeal Sec complex is more homologous to eukaryotes then to bacteria. The α-subunit contains 10 transmembrane (TM) alpha-helices divided into two domains that contain TMs 1-5 and TMs 6-10. The γ- and β-subunits span the membrane once each. Visualization of the Secαγβ complex from the cytoplasmic side reveals the α-subunit as a rectangular shape with three of its four sides surrounded by the γ- and β-subunits (van den *et al.*, 2004). One single α-subunit (SecY) forms an hourglass-shaped translocation channel, which is plugged by one of its short alpha-helices. Binding of a signal peptide is thought to result in displacement of the plug and opening of the channel. As the channel expands and the signal peptide intercalates between TM2 and TM7, the mature portion of the polypeptide moves through the aqueous channel. The hydrophobic residues in the channel constriction may form a gasket-like seal around the polypeptide as it translocates through the channel thus preventing the passage of other molecules. Once the translocating polypeptide exits the channel, the plug returns to its original position. The structure of the Sec complex also suggests a model for transport of membrane proteins (van den *et al.*, 2004). The transmembrane domain of membrane proteins is thought to exit the channel laterally to partition into the lipid bilayer. As the α-subunit channel is blocked on three of its four sides by the γ- and β-subunits, the free side is likely the place where the channel may open to allow for escape of membrane proteins.

Not all Sec dependent precursors engage the translocase through interactions with SecB. It is generally thought that resident inner membrane proteins require a divergent method of entry into the Sec pathway. There are two pathways known to exist in *E. coli*. One requires targeting of ribosome-nascent chains (RNC) through interactions with the signal recognition particle (SRP). SRP carries the nascent chain to the Sec translocon, which is associated with YidC. The other pathway completely bypasses the Sec translocon and directly targets RNC to YidC (Luirink and Sinning, 2004) (de Gier and Luirink, 2003).

### *Twin-arginine translocation (Tat) pathway*

The Sec pathway supports export of unfolded proteins across the inner membrane. However several bacterial proteins that incorporate cofactors such as NADP, metal ions like copper and complex metal cofactors (e.g. molybdopterin) must do so as they fold into their native state. Cofactors normally reside in the cytoplasm. Therefore cofactor-containing proteins must fold in the cytoplasm before they are exported across the inner membrane. Pre-folded proteins can not utilize the Sec machinery for transport. A recently described system, the twin-arginine transport (Tat) pathway, distinct from the Sec apparatus, recognizes and exports folded proteins across the membrane (Robinson and Bolhuis, 2004). In addition to cofactor-containing proteins, the Tat machinery also transports other proteins such as phospholipase, a virulence factor secreted by *Pseudomonas aeruginosa* (Ochsner *et al.*, 2002). A similar system was recognized first in the thylakoid membrane of plant chloroplasts (Settles *et al.*, 1997).

Evidence indicates that Tat substrates are recognized and sorted via a signal peptide. The signal sequence varies in length (up to 58 amino acids) but contain common features. The signal peptide has three domains, a basic amino-terminal region (n-region) followed by a hydrophobic h-region and a hydrophilic domain (c-region) that contains the recognition site for the enzyme signal peptidase (Berks, 1996). In addition, the Tat signal peptides contain a conserved amino acid sequence motif at the n-region/h-region site which is defined as S-R-R-x-F-L-K (Stanley *et al.*, 2000). Although the vast majority of Tat substrates carry the twin arginine motif within the signal sequence, site-directed mutagenesis show that not all Tat translocation competent proteins require the presence of the arginine pair (Dreusch *et al.*, 1997) (Gross *et al.*, 1999) (Halbig *et al.*, 1999). In fact the Tat pathway exports an *E. coli* penicillin amidase even though it has no obvious twin-arginine-like motif (Ignatova *et al.*, 2002). The signal peptide sequence requirement for Tat sorting and transport has been explored by a series of fusion protein studies. For example, a fusion between the Sec-dependent c-cytochrome and a Tat signal peptide diverted this recombinant protein to fold in the cytoplasm and to be recognized and transported by the Tat pathway (Sanders *et al.*, 2001). All together evidence gleaned by several investigators reinforces the concept that the entire signal sequence in context with the rest of the protein may well be important and not just the arginine motif (Robinson and Bolhuis, 2004). Furthermore, heterologous signal sequences may not be recognized as Tat substrates by unrelated bacteria (Berks *et al.*, 2000a). For example, while glucose-fructose oxidoreductase (GFOR) of *Zymomonas mobilis* is exported by the Tat pathway in its native host it is not exported when expressed in *E. coli* (Blaudeck *et al.*, 2001). Yet when the *Z. mobilis* signal sequence was replaced by the signal sequence used to target an *E. coli* enzyme, trimethylamine N-oxide reductase (TorA), the GFOR-fusion protein was readily exported by *E. coli* (Blaudeck *et al.*, 2001).

Recent studies indicate that soluble chaperons, proteins that bind newly synthesized polypeptide chains to prevent incorrect folding, may carry Tat substrates to the machinery for export. These chaperons may have a proofreading function that rejects unfolded and incorrectly folded proteins (Robinson and Bolhuis, 2004), and proteins that do not bear the proper signal sequence thereby blocking their entry into the Tat pathway (DeLisa *et al.*, 2003) (Jack *et al.*, 2004). One example of a recently discovered putative molecular chaperone is DmsD. This protein specifically binds to the precursor form of dimethylsulfoxide reductase (Ray *et al.*, 2003) prior to its interaction with the Tat translocase. DmsD was shown not to bind the mature form of the DmsA protein (Papish *et al.*, 2003) and in a strain lacking DmsD the biogenesis of DmsA was severely affected (Oresnik *et al.*, 2001) (Ray *et al.*, 2003). DmsD appears to assist in proper folding and targeting of the DmsA precursor to the Tat apparatus (Papish *et al.*, 2003). As of yet no global chaperons have been identified that participate in Tat substrate biogenesis (Robinson and Bolhuis, 2004).

### *Characteristics of the components of the Tat translocase*

Four gene products, TatA, TatB, TatC, and TatE, have been identified that participate in Tat export (Weiner *et al.*, 1998) (Sargent *et al.*, 1998) (Bogsch *et al.*, 1998) (Bolhuis *et al.*, 2001). Generally TatAB and C are found in most Gram-negative bacteria but there is some variability amongst Gram-positive bacteria (Robinson and Bolhuis, 2004). In *E. coli, TatABC* are located in a polycistronic locus on the chromosome whereas *tatE* has a different chromosomal location (Robinson and Bolhuis, 2004). TatE and TatA are homologous proteins with approximately 50% sequence identity and *tatE* may be a gene duplication of *tatA* (Robinson and Bolhuis, 2004). Genetic evidence indicates that TatE and TatA have overlapping functions because single deletions of either TatA or TatE decrease the number of Tat-dependent proteins exported but does not abolish it. The reason for the redundancy of function for TatA and TatE in *E. coli* is unclear since some species of bacteria do not carry the *tatE* gene at all and those that do so express TatA at much higher levels than TatE (Sargent *et al.*, 2001) (Jack *et al.*, 2001). TatA, TatB, and TatE are integral membrane proteins each with one transmembrane domain (TM) (De *et al.*, 2001). TatC is predicted to have 6 TMs (Sargent *et al.*, 1998) (Bogsch *et al.*, 1998), but recent data indicates that TatC may have only 4 TMs (Drew *et al.*, 2002) (Jack *et al.*, 2004).

Evidence indicates that the Tat pathway relies on the transmembrane proton electrochemical gradient across the inner membrane for protein export (Robinson and Bolhuis, 2004). The stoichiometry and composition of the functional Tat complex is under investigation.

Tat substrates are thought to be exported across the inner membrane through a gated pore. TatA is a good candidate to form the protein translocation channel because it is expressed in large molar excess over other Tat components. TatA forms oligomers and is not involved in signal peptide-binding. Furthermore, negative stain electron microscopy of isolated TatAB complexes revealed that TatA was in 25 molar excess and formed ring-like structures that contained a central pore of approximately 70Å. These results are very

provocative since inspection of folded Tat substrates indicates that in order to transport them the channel must be at least 70Å wide (Berks *et al.*, 2000b) (Sargent *et al.*, 2002). Finally, in support of these findings antibodies to the TatA orthologue, Tha4 of plant chloroplasts, prevent protein transport but does not block its association with precursor proteins (Cline and Mori, 2001) indicating that TatA does not participate in substrate recognition but functions in protein export. The totality of evidence to date suggests a model in which the TatBC complex functions to bind substrate while the large TatA complex forms the pore in association with TatBC to export the prefolded protein across the inner membrane (Robinson and Bolhuis, 2004). The exact mechanism of protein translocation and the precise configuration of the Tat machinery remain to be discovered.

## OUTER MEMBRANE TRANSLOCATION

### *Autotransporters: Members of the type V secretion system*

Many pathogenic microorganisms use the simplicity of the type V pathway for extracellular transport of effector molecules, which have profound effects on eukaryotic cells. This diverse group of effector proteins consists of the cytolytic serine protease autotransporters secreted by the Enterobacteriaceae, adhesive type proteins such as pertactin of *Bordetella pertussis*, phospholipase of *P. aeruginosa*, and VacA of *Helicobacter pylori*. Autotransporters are a large family of diverse proteins produced by Gram-negative bacteria specifically the α-β-γ and ε classes of proteobacteria (except *Actinobacillus* and *Vibrio*), and by *Chlamydiae*. The autotransporter secretion modality was first described in 1987 for the IgA1 protease produced by *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987). Since then several others have been identified. Only pathogenic bacteria appear to produce these autotransporters. In some cases a direct link to the pathology of the disease can be correlated as for the vacuolating toxin (VacA) of *H. pylori* (Reyrat *et al.*, 1999) and the IcsA autotransporter important for intracellular motility of *Shigella flexneri* (Goldberg and Sansonetti, 1993). In many cases however, no definitive role in the disease process can be attributed to the autotransporter even when its function is known (Henderson and Nataro, 2001).

Autotransporters are unique because they contain, programmed into their structure, all the information required for self-transport across the outer membrane without accessory proteins. These proteins are synthesized as large pre(pro)proteins with three distinct regions: a Sec- sorting amino terminal signal peptide, a passenger domain (effector region of the protein) and a C-terminal β-domain dedicated for outer membrane translocation of the passenger domain. Once synthesized, the autotransporters are transferred across the inner membrane in a sec-dependent manner. Following removal of the leader peptide, the (pro)protein is released into the periplasm and self-translocated across the outer membrane (Desvaux *et al.*, 2004a). The primary features of an autotransporter are discussed below.

Some autotransporters contain an 18-27 amino acid residue leader peptide (Desvaux *et al.*, 2004a). Others have an atypical Sec sorting sequence that is unusually long (up to 50 residues). The signal sequences display three distinct features: the n-domain contains residues with a positive charge; a hydrophobic group of residues designated as the h-domain; and consensus signal peptidase recognition site that consist of the c-domain. In addition, these sorting sequences contain at the beginning of the n-domain two specific motifs: MNKIYSLKY (S/C/H) followed by conserved hydrophobic residues, GLIAVSELAR (Oliver *et al.*, 2003a) (Desvaux *et al.*, 2004a). Cross-linking experiments between the hemoglobin protease autotransporter (Hbp) and components such as SRP, trigger factor, SecA, and SecY, along with pulse chase and mutational analysis of the Hbp leader sequence yielded evidence that the additional residues were sorting sequences for recruitment of SRP. In fact, this was the first time an outer membrane protein was shown to use the SRP-Sec pathway to cross the inner membrane (Sijbrandi *et al.*, 2003).

After the autotransporter reaches the periplasm the hydrophobic residues of the C-terminal β-domain interacts with the lipid bilayer of the outer membrane to initiate self-insertion (Henderson *et al.*, 1998). A consenus motif (Y/V/I/F/W)X(F/W) at the end of the β-domain appears to be extremely important for folding, formation and stabilization of the translocation pore. The β-domains form β-barrels that consist of 14 antiparallel amphipathic strands consisting of 9-12 residues (Desvaux *et al.*, 2004a). The translocation pore forms an aqueous channel such that the hydrophobic domains of the alternating residues are embedded into the lipid bilayer of the outer membrane and the hydrophilic residues face toward the center of the pore. The exact configuration of the pore in the outer membrane is under debate. Some evidence suggests that the pore is formed by oligomers of the β-domain (Veiga *et al.*, 2002), but this structure is still speculative as other studies point to a monomeric conformation (Oomen *et al.*, 2004).

As the β-domain begins to fold into a β-barrel, its most stable and favorable conformation, the tethered

unfolded passenger domain is exposed to the periplasmic space (Desvaux *et al.*, 2004a). That situation makes this domain susceptible to proteases unless pore formation and passenger domain translocation are temporally coupled so that the periplasmic stage is short-lived. Recently it was hypothesized that a periplasmic chaperone aided the translocation process. Indeed a study by Voulhox and colleagues revealed that depletion of the periplasmic chaperone Omp85 resulted in poor cell viability and resulted in significant reduction of processed IgA1 protease on the cell surface. Taken together the data suggests that Omp85 plays a crucial role in the biogenesis of outer membrane proteins (Voulhoux *et al.*, 2003).

A linker region located between the passenger domain and the β-domain is essential for threading the unfolded passenger domain through the pore of the transporter (Oliver *et al.*, 2003b) (Velarde and Nataro, 2004). Once at the cell surface, the passenger domain folds (Ohnishi *et al.*, 1994). A short amino acid segment present in the passenger domain was found to act as a molecular chaperone (even when supplied in trans) to trigger or direct folding of the effector domain (Ohnishi *et al.*, 1994) (Oliver *et al.*, 2003b).

After folding, the effector molecule can remain intact and stay associated with the β-domain or may be cleaved. The cleavage occurs upstream of or within the linker region by proteolytic activity associated with the autotransporter itself (Serruto *et al.*, 2003) or by an exogenous membrane associated protease (Shere *et al.*, 1997). Although cleaved, some proteins remain noncovalently associated with the β-domain. In other cases, further maturation of cleaved and released protein can occur to achieve an active state. For example, VacA is cut into two subdomains, one 33 kDa and the other 55 kDa. These two subunits oligomerize into a large functional complex (Lupetti *et al.*, 1996).

### *Two-partner secretion pathway*

The two-partner secretion pathway contains members of a subgroup of the type V secretion family. Extracellular transport of these proteins is similar to other members of the autotransporter family with a few differences summarized here. The two-partner secretion system is distinctive in that the effector protein (secreted protein) and the transporter are the products of two separate genes. These genes are located in an operon or can be found at different loci (Jacob-Dubuisson *et al.*, 2001). Each transporter is dedicated to transport of one specific exoprotein. The secreted proteins can be grouped by their functions although there are limited identifiable similarities within the secretion domain of these proteins (Jacob-Dubuisson *et al.*, 2001). The effector proteins are produced as large pre(pro)-proteins which transverse the inner membrane in a Sec-dependent manner. The transporter has dual functions; it transports the pro-proteins released into the periplasm and acts as a chaperone to prevent premature folding prior to translocation. Once at the cell surface the proteins fold, followed by processing when necessary to yield functional proteins (Jacob-Dubuisson *et al.*, 2001).

## The type II secretion pathway

The type II secretion system (T2SS) has also been referred to as the main terminal branch of the general secretion pathway because in the past it was believed to be the main route across the outer membrane (Pugsley *et al.*, 1997) (Pugsley, 1993). Over time the discovery that only a subset of proteins produced by Gram-negative bacteria are secreted by the type II machinery has revised this assumption (Desvaux *et al.*, 2004b). Even so, many pathogenic and non-pathogenic Gram-negative bacteria produce functional T2SSs to transport proteins across the outer membrane. Since the identification of the T2SS as crucial for secretion of pullulanase by *Klebsiella oxytoca* (d'Enfert *et al.*, 1987) (d'Enfert and Pugsley, 1989) several human pathogens such as *Vibrio cholerae*, *Yersinia pestis*, Shiga-toxin producing *E. coli*, *P. aeruginosa*, and plant pathogens like *Erwinia chrysanthemi* and *Xantomonas campestris* have been shown to have adapted this method for secretion of key virulence factors such as toxins, proteases, chitinases, lipases, and other hydrolytic enzymes out of the cell (Sandkvist, 2001b) (Sandkvist, 2001a). The T2SS has also been identified in *Legionella pneumophila* where it promotes growth of Legionella at temperatures at or below 30°C (Soderberg *et al.*, 2004). It is suggested that a diffusible factor secreted by the type II machinery is required for survival of Legionella at those temperatures.

Secretion via the type II machinery occurs, just like secretion via the type V pathway, in two distinct stages (Hirst and Holmgren, 1987a). First, nascent polypeptides destined for transport cross the inner membrane via the Sec or Tat pathway (Filloux *et al.*, 1990) (Voulhoux *et al.*, 2001), the signal peptide is cleaved and the proteins are released. Second, after entry into the periplasm exoproteins fold and assemble into their native or near- native states. Lastly, these substrates engage the type II secretion apparatus (secreton) in the periplasmic compartment prior to translocation across the outer membrane.

### *Substrate recognition by the type II machinery.*

Type II-dependent proteins secreted by various species of bacteria are quite divergent in function with very little similarity at the amino acid level. As an example, *V. cholerae* secretes 6 distinct proteins that include

cholera toxin and hemagglutinin-protease. Cholera toxin is a multisubunit protein that consists of 5 identical B subunits (the binding domain) and one A subunit (catalytic domain) that fold and assemble in the periplasm into $AB_5$ holotoxin with the help of disulfide isomerase (DsbA) (Findlay *et al.*, 1993) prior to translocation across the outer membrane. On the other hand hemagglutinin-protease secreted by *V. cholerae* is a monomeric protein. Notwithstanding these differences, the type II secretion machinery discriminates between resident periplasmic and secreted proteins and allows only type II dependent substrates to enter into the secreton. How are type II-dependent proteins selected? Information gleaned from mutational analyses of amino acid residues at the amino and carboxy-terminus of secreted products has been ambiguous and did not distinguish residues that were identifiable as signal sequences but suggested that residues at both ends were important and might include two different signals that act synergistically (McVay and Hamood, 1995) (Lu and Lory, 1996). Internal deletions of exotoxin A that preserved the functional domains of the protein were secretion defective (Voulhoux *et al.*, 2000). Likewise internal deletions or insertions within cellulose (Chapon *et al.*, 2001) that preserved function prevented secretion by the *Erwinia* type II machinery (Py *et al.*, 1993). When disulfide bond formation, which normally occurs in the periplasm, was blocked cellulase (Chapon *et al.*, 2001) and cholera toxin were not secreted by their cognate type II machinery but accumulated in the periplasm and were eventually degraded (Bortoli-German *et al.*, 1994) (Shevchik *et al.*, 1997) (Hirst and Holmgren, 1987b) (Hardy *et al.*, 1988). On total these experiments indicated that accurate folding in the periplasmic compartment is crucial for secretion. Analysis of exotoxin A and elastase supports the premise that, exclusive of disulfide bond formation proper periplasmic folding is a prerequisite for type II-dependent secretion and that a secretion motif is likely presented by the three-dimensional structure of the substrate (Braun *et al.*, 1996) (Lu and Lory, 1996) (Sauvonnet and Pugsley, 1996). Even distant residues that converge after folding may play a role in forming a structural secretion motif (Voulhoux *et al.*, 2000) (Chapon *et al.*, 2001). The *E. coli* heat-labile enterotoxin (LT-II) B subunit shares only 11 % identity with the B subunit of cholera toxin yet when expressed in *V. cholerae* it was recognized and secreted via the T2SS (Connell *et al.*, 1995). Comparison of the molecular structure of the B subunits of cholera toxin and LT-II reveals that the structures are nearly identical which probably explains why LT-II B was secreted by *Vibrio*. The pivotal finding by Connell and coworkers showed that conformation of the substrate is crucial to secretion competence. Yet to date no conspicuous three-dimensional motif has been identified by sequence analysis, chimeric protein technology or mutational analysis. One caveat certain to be considered is that chaperon(s) may be involved in presentation of substrates to the secretion apparatus. For instance the membrane anchored lipase-specific foldase, Lif, forms a complex with *P. aeruginosa* lipase to aid its folding, recognition, uptake and secretion by the type II machinery (El *et al.*, 1999).

Substrates transported by one species of bacteria by and large can not be secreted by an unrelated species, but this stringent restriction can be overcome when closely related species are considered. For example the *K. oxytoca* pullulanase can not be secreted by *P. aeruginosa* (de *et al.*, 1991) but lipase of *P. alcaligenes* is recognized and secreted by *P. aeruginosa* (de *et al.*, 2001).

### *The type II machinery: a supramolecular complex*

The species of bacteria that produce type II secretion apparatus are quite diverse and as a consequence the number of genes necessary for type II secretion varies from 12 to 15. These genes are designated as A-O and S (except in *Pseudomonas* where the genes are designated Q-Z and O). See Sandkvist for more details (Sandkvist, 2001a). The type II genes C-N generally cluster in an operon and the arrangement of those genes within the operon is fairly conserved (Sandkvist, 2001a). There is some variability as to which genes are essential for secretion depending on which species of bacteria is considered. Of note, when genes encoding the T2SS of *K. oxytoca* were transformed into laboratory strains of *E. coli* all but pulB, pulH, and pulN were found to be essential for secretion of pullulanase (Possot *et al.*, 2000). The A and B genes are not present in all species and variability in the need for A and B gene products have been noted for *Erwinia* and *Aeromonas* spp. (Howard *et al.*, 1996) (Jahagirdar and Howard, 1994). Protein S is thought to be important for stability and outer membrane insertion of protein D but may only be essential for secretion in *Erwinia* and *K. oxytoca* (Shevchik *et al.*, 1997) (Hardie *et al.*, 1996). In contrast, the O gene product is essential in all species tested thus far and is required for processing of proteins G, H, I, J, and K (Strom *et al.*, 1993) (Nunn and Lory, 1993) (Bleves *et al.*, 1998).

Various techniques such as mutational analysis, co-immunoprecipitation, yeast-two hybrid, cross-linking studies, and hunt for suppressor mutations have identified many protein-protein interactions and recognized the function for some key proteins of the T2SS (Sandkvist, 2001b). In addition, fractionation experiments have determined the subcellular location for most of these components. Based on localization data

and intermolecular interactions the evidence supports the hypothesis that a supramolecular complex is formed and that this apparatus spans the cell envelope from the inner membrane to the outer membrane (see Figure 5.2).

### *Protein D: the type II secretin*

It is predictable that the translocation pore would have to be large in view of the fact that proteins transported through the type II machinery are folded prior to secretion. The D gene product is an integral outer membrane protein believed to be the translocation pore (Akrim *et al.*, 1993) (Bitter *et al.*, 1998). It forms a homomultimeric complex and the C-terminal portion of the protein appears to be important for oligomerization (Bitter *et al.*, 1998). Electron microscopic analysis revealed that both the intact D protein and its isolated C-terminal domain form hexagonal-like ring-shaped oligomers that consist of 12-14 monomers (Linderoth *et al.*, 1997) (Brok *et al.*, 1999) (Nouwen *et al.*, 2000). D multimers have a central pore size of approximately 95 Å, large enough to accommodate pre-folded proteins such as elastase which has a diameter of 60 Å when folded (Thayer *et al.*, 1991) (Bitter *et al.*, 1998). The pore must be gated to preserve the membrane integrity of the cell but the mechanism of gating is unknown (Brok *et al.*, 1999). The C-terminal domain is embedded within the outer membrane and forms a 13 stranded β-barrel in a manner reminiscent of other integral outer membrane proteins (Bitter *et al.*, 1998). The N-terminal domain extends into the periplasm where it may contact other components of the type II machinery (Brok *et al.*, 1999). There are normally 50-100 complexes per cell as estimated by semi-quantitative immunoblotting (Brok *et al.*, 1999). The C-terminal portion of the D protein shares a high level of identity to a large family of proteins called secretins. Secretins are not only critical for type II secretion but are also important for biogenesis of type IV pili, surface appendages that play important roles in pathogenesis and participate in twitching motility, social gliding, and biofilm formation. Other members of the secretin family are involved in type III secretion and filamentous phage extrusion.

### *The NTPase of the type II apparatus*

Protein E associates peripherally with the cytoplasmic membrane in the presence of protein L (Sandkvist *et al.*, 1995) (Ball *et al.*, 1999) (Py *et al.*, 1999) (Possot *et al.*,

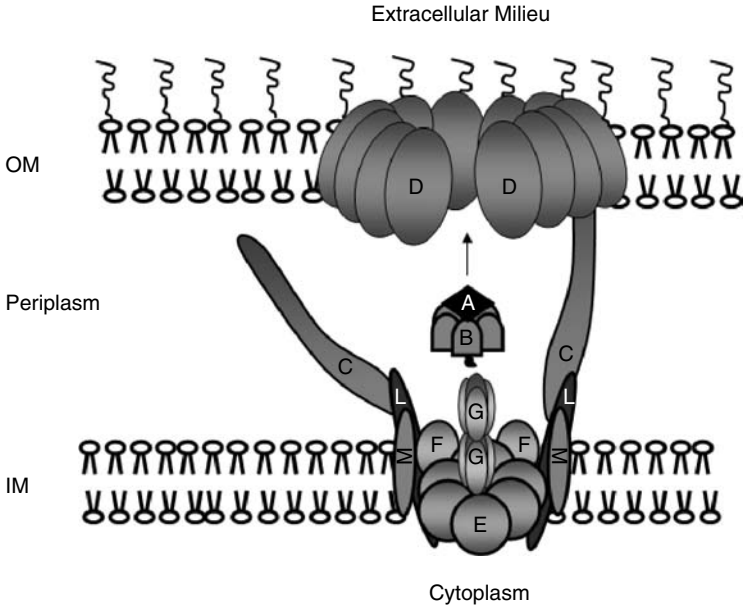



**FIGURE 5.2** A model of *c*holera *t*oxin (CT) secretion by the type II secretion pathway. Individual precursor subunits of cholera toxin are exported across the inner membrane by the Sec pathway and then the signal sequence is removed. In the periplasm the individual A and B subunits fold, assemble into holotoxin with the help of disulfide isomerase DsbA and the holotoxin is then targeted to the secretion machinery by way of the B subunit. The C protein with D may gate the entrance to the secretion pore. Following engagement by CT conformational changes in the apparatus facilitate translocation of the toxin. Secretion may proceed as a result of polymerization of component G and other pilin-like proteins into a piston-like structure that extends from the E/F/L/M platform found in the inner membrane. Protein E may be crucial for regulation or energizing polymerization of the pilin subunits so that CT is translocated across the outer membrane. This model does not include proteins A, B, N, and S because they are not present in all every organism that encode and produce the type II apparatus. IM refers to the inner membrane and OM to the outer membrane.

2000). It is believed to be the energy- generating component of the T2SS (Sandkvist, 2001b) and recently it was unequivocally shown that *V. cholerae* protein E (EpsE) possesses ATPase activity (Camberg and Sandkvist, 2005). The crystal structure of EpsE that contained an N-terminal truncation was determined and revealed a two-domain structure (Robien *et al.*, 2003). The C-terminal domain containing the C1 subdomain displays characteristic features noted for other $AAA^+$ ATPases. Namely, the Walker A box necessary for nucleotide binding, an atypical Walker B box and also present in between the Walker boxes the aspartate-rich motif. Furthermore, downstream of the Walker B box the histidine box was identified along with a unique motif that contains a protruding loop that forms when four cysteines come together to coordinate a zinc ion (Robien *et al.*, 2003;Camberg and Sandkvist, 2005). Protein E is a member of the ATPase subfamily that includes distant relatives VirB11 and HP0525 that participate in type IV secretion. Previous structural and electron microscopy data showed that members of the VirB11 subfamily such as TrwB and HP0525 form hexameric rings. The structural homology between HP0525 and EpsE supports the assertion that EpsE forms homo-hexamers and suggests that the hexamer may be the physiologically relevant form of EpsE that interacts with the rest of the type II complex (Robien *et al.*, 2003).

#### *Pseudo-pilin components: G, H, I, J, and K*

Amino acid similarity at the N-terminus indicates that type II pseudo-pilins have a common ancestry with pilin subunits of the type IV pilus system and exhibit similar properties. As a matter of fact, the major pseudo-pilin protein, G can polymerize when over expressed to form pilus-like structures (Sauvonnet *et al.*, 2000) (Durand *et al.*, 2003). Protein G interacts with H, I, and J, and interactions with E have also been suggested (Kagami *et al.*, 1998) (Possot *et al.*, 2000). Consequently one model suggests energy supplied by protein E might drive polymerization of the major pilin-like component, G, to form a pilus-like structure to propel substrates through the outer membrane pore (Sandkvist, 2001b) (See Figure 2). The roles of H, I, J, and K are not known. Recently yeast-two hybrid system revealed interactions of J with proteins G, D, L, and I (Douet *et al.*, 2004).

#### *Inner membrane subassembly*

Proteins C, L, and M have no recognizable homologues in the type IV pilus biogenesis system (Peabody *et al.*, 2003) and may be unique to T2SS. Protein C can be found in both inner and outer membrane fractions, and cross-linking studies show that it forms stable contacts with D (Possot *et al.*, 1999). Most other components of the type II apparatus are found in the inner membrane, and well documented evidence indicates that these interacting partners include E, L, M, and F; [E and L interactions: (Sandkvist *et al.*, 1995), (Py *et al.*, 1999), (Ball *et al.*, 1999), (Possot *et al.*, 2000)] [L and M interactions (Michel *et al.*, 1998), (Sandkvist *et al.*, 1999), (Possot *et al.*, 2000)] [E,L, and F interactions (Py *et al.*, 2001)]. Protein C may interact with both L and M. Taken together the data imply that protein C probably connects the outer membrane secretion pore (protein D) with the rest of the inner membrane type II components thereby forming the supramolecular complex that spans both inner and outer membranes.

## Type IV secretion pathways

The type IV secretion system (T4SS) comprises three distinct subgroups, which share structural homologies but secrete different substrates. One subgroup includes systems involved in DNA transfer such as the conjugal system found in both Gram-negative and Gram-positive bacteria. Conjugative transfer of DNA is an effective method of spreading antibiotic resistance genes carried on conjugative plasmids between related and unrelated bacteria (Lawley *et al.*, 2003). Another form of conjugation system transfers genetic material among organisms from different kingdoms. Such a system was first identified in the phytopathogen *Agrobacterium tumefaciens* as a means of transferring oncogenic T-DNA directly into susceptible plant cells (Zupan *et al.*, 2000). The second subgroup consists of DNA uptake and DNA release systems in which transfer does not require immediate contact with a target cell. This DNA uptake modality is used by *Campylobacter jejuni* and *H. pylori* to provide a method of genetic transformation between related species of bacteria to augment pathogenesis (Hofreuter *et al.*, 2001). *N. gonorrhoeae* is infamous for its capacity to undergo horizontal gene transfer between species and encodes a type IV secretion system involved in DNA release (Dillard and Seifert, 2001). The third subgroup has evolved to support translocation of proteinaceous virulence factors directly from the cytosol of pathogenic bacteria into human or animal host cells. The pathogens include both intracellular and extracellular organisms such as *L. pneumophila*, *Brucella* spp., *Bartonella* spp. and *H. Pylori,* which rely on effector molecules secreted by the type IV machinery for virulence and survival (Cascales and Christie, 2003). The T4SS of *Bordetella*, Ptl, employs a variation of this scheme and does not require cell contact because pertussis toxin (a type IV substrate) is released into the environment, binds to its receptor and is then internalized by the host cell (Burns, 2003).

### *Gene products necessary for type IV translocation and regulation*

The most well characterized T4SS is Vir carried by *A. tumefaciens*, and consequently this system will be the focus of the discussion on type IV mediated secretion. Comparisons will be made to other systems where appropriate. The T4SS of *A. tumefaciens* consists of 11 products of the *virB* and *virD* operons. The gene products VirB2–11 are required for assembly of a cell envelope spanning complex, for T-pilus formation, and for substrate transfer. The protein channel and the T-pilus together are designated as the mating pair formation [Mpf] (Cascales and Christie, 2003). The DNA transfer replication (Dtr) proteins, VirD1 and VirD2, process DNA substrate (Shirasu *et al.*, 1994) for transfer (Cascales and Christie, 2003). VirD4, referred to as the coupling protein (CP), is important for T-DNA recognition and targeting to the Mpf structure for translocation (Cascales and Christie, 2003).

The expression of *virB* and *virD* genes is regulated by environmental signals. Wound released phenolic compounds such as acetosyringone and sugars like monosaccharides activate the two-component regulator system of which VirA is the sensor and VirG is the transcription factor (Chang and Winans, 1992). Other factors such as phosphate starvation and extracellular pH below 6 also activate the two-component system, which in turn induces the expression of the *virB* and *virD* genes. In the case of *L. pneumophila* and *Brucella* spp., VirB homologues may be induced by amino acid depletion and acidic pH as a consequence of host cell internalization (Bachman and Swanson, 2001) (Rouot *et al.*, 2003). In contrast, the pertussis toxin (*ptx*) genes and the *ptl* genes carried by *B. pertussis* are co-transcribed as they share a common promoter upstream of *ptx* (Ricci *et al.*, 1996). Ptx has been shown to be expressed following an increase in temperature from 25°C to 37°C and is probably induced upon entry of bacteria into the host (Melton and Weiss, 1989).

### *Recruitment of type IV substrates*

Protein translocation by the T4SS begins with recruitment of cognate DNA or protein substrates, continues with transfer of substrate to the secretion machinery and ends with translocation across the bacterial cell envelope. In most cases effectors are delivered directly to target cells following cell-to-cell contact during infection (Cascales and Christie, 2003). The exact mechanism of substrate recognition by the CP and exactly how type IV substrates are transferred to the translocation machinery is not known. There has been no distinct transport signal identified but protein fusion data indicates that the C-terminal region contains residues important for translocation (Vergunst *et al.*, 2000) (Schrammeijer *et al.*, 2003). For example, fusion proteins composed of Cre/loxP and C-terminal portions of RalF or LidA, two proteins secreted by the T4SS Dot/Icm in *L. pneumophila*, but not N-terminal fusions of these proteins were competent for translocation into recipient cells (Luo and Isberg, 2004). Other findings from studies of the *A. tumefaciens* effector proteins VirE2, VirE3, and VirF, suggest that a cluster of positively charged amino acids present within the last 30-50 residues of the C-terminus play a role in defining the recognition signal (Vergunst *et al.*, 2003). Experimental evidence suggest that the function of the coupling protein, VirD4, is to interact with and bind substrate (T-DNA or effector molecules) and transfer these materials from the cytoplasm to the Mpf complex for translocation across the cell envelope (Llosa *et al.*, 2002). A novel cytological fluorescent screen in conjunction with immunoprecipitation analysis revealed direct interactions between the substrate, VirE2, and VirD4 indicating that substrates are recruited to the coupling protein (Atmakuri *et al.*, 2003). All the evidence taken together indicates that the role of coupling proteins is to serve as a connector between the proteins (or DNA) to be secreted and the secretion apparatus (Llosa *et al.*, 2003). This finding may be important to understanding effector translocation by other pathogenic bacteria such as *H. pylori* and *L. pneumophila* that also utilize CPs to recruit substrate to the translocation machinery (Cascales and Christie, 2003). In contrast the *Bordetella* Ptl system bypasses the CP step and uses the Sec pathway for export of Ptx subunits across the inner membrane (Cascales and Christie, 2003). Likewise *Brucella* and *Bartonella* do not use a CP mechanism for the recruitment of substrates to the secretion machinery, but the method used is unknown (Cascales and Christie, 2003).

### *Components of the type IV machinery*

Various methods employed in studying components of the type IV apparatus have reinforced the belief that the type IV assembly is a multi-protein complex that spans the cell envelope (Cascales and Christie, 2003). In deed use of a novel method called transfer DNA immunoprecipitation (TrIP) showed that the T-DNA/VirD2 complex interacts in a sequential manner with the VirD4 coupling protein, VirB11 ATPase, and the inner membrane channel components VirB6 and VirB8 and exiting byway of contacts with the secretin and pilin proteins, VirB9 and VirB2 (Cascales and Christie, 2004). The totality of evidence available has allowed a picture of the apparatus to take shape (Figure 5.3).

### *Coupling protein*

As discussed above CP is crucial for recruitment of substrates to some, but not all T4SSs. The CP is an inte-

gral membrane protein that contains two membrane-spanning domains at its N-terminus, a small periplasmic region and a significant portion of the C-terminus protruding into the cytoplasm (Cascales and Christie, 2003). The N-terminal portion of the protein is important for homo-oligomerization and X-ray crystallography of a VirD4 homolog from *H. pylori* indicates that CPs may transition between monomeric and hexameric states as a consequence of substrate or nucleotide binding (Cascales and Christie, 2003).

### *Energy couplers: VirB11 and VirB4*

VirB11 associates peripherally with the cytoplasmic face of the inner membrane (Cascales and Christie, 2003). This protein belongs to the superfamily of NTPases that function in various secretion systems present in both Gram-negative and Gram-positive bacteria and Archaea (Cascales and Christie, 2003). X-ray crystallography and electron microscopy comparisons of the VirB11-like type IV proteins, TrwB and HP0525, suggest a hexameric pore conformation (Gomis-Ruth and Coll, 2001) (Gomis-Ruth *et al.*, 2002) (Yeo *et al.*, 2000). The opening and closing of the pore appears to be regulated by ATP binding and hydrolysis resulting in dynamic conformational changes that may drive substrate across the inner membrane or aid the assembly of the translocation machinery (Savvides *et al.*, 2003). Recent information indicates that substrate is transferred from VirD4 to VirB11 in a process that does not require ATP binding or hydrolysis (Atmakuri *et al.*, 2004). On the other hand subsequent transfer of substrate from VirB11 to core proteins of the channel, VirB6 and VirB8, is driven by ATP hydrolysis (Atmakuri *et al.*, 2004).

VirB4 is an integral membrane protein that possesses two transmembrane domains and may function as a homomultimer (Rabel *et al.*, 2003). It also contains a centrally located Walker A box however ATP hydrolysis has not yet been demonstrated (Dang and Christie, 1997). Yeast-two hybrid analysis indicates that VirB4 and VirB11 interact (Ward *et al.*, 2002), suggesting that these two proteins may cooperate to drive substrate translocation. However the exact function of VirB4 has not been determined.

### *Inner membrane components: VirB6, VirB8, and VirB10*

These proteins have no known homologues outside of type IV secretion systems (Cascales and Christie, 2003) and probably forms the inner membrane portion of the secretion channel. VirB8 brings together a subcomplex that consists of itself, VirB9 and VirB10 and without VirB8, interactions between VirB9 and VirB10 not occur

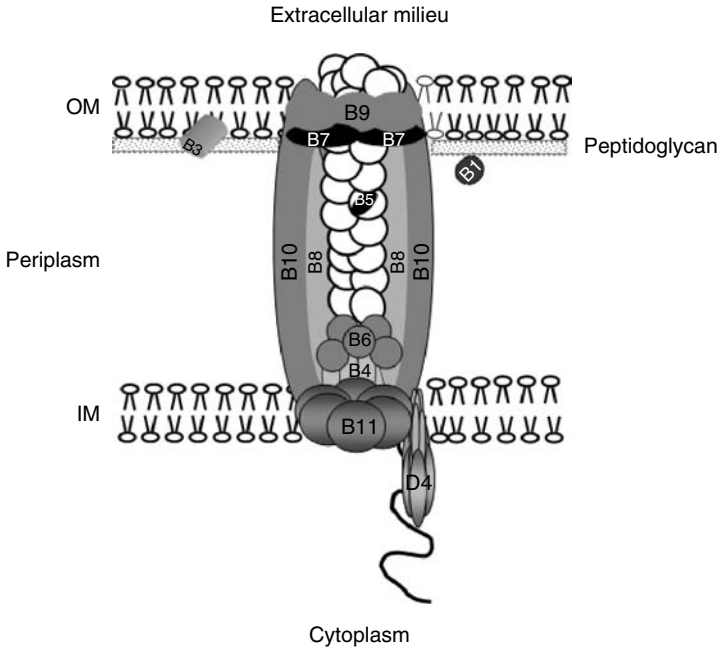



**FIGURE 5.3** Illustration of the *A. tumefaciens* type IV secretion apparatus. This rendering of the structure as a multimolecular membrane-spanning apparatus is based on recent data obtained from yeast or bacterial dihybrid screens and other biochemical analyses. The stoichiometry of the components that cooperate to form the secretion apparatus and the exact configuration of the structure within the lipid bilayer is not known. Molecules are not drawn to scale. VirB1 is not part of the macromolecular complex but is a transglycosylase that degrades the peptidoglycan layer and aids assembly of the pilus. VirB3 is an outer membrane protein thought to stabilize assembly or function of the apparatus. Inner membrane (IM) and outer membrane (OM) are shown.

(Christie, 2004). Both VirB8 and VirB10 are bitopic integral membrane proteins with large C-terminal periplasmic domains (Christie, 2004). The VirB6 constituent has 5 transmembrane spanning domains with the N-terminus located in the periplasm and the C-terminus in the cytoplasm (Jakubowski *et al.*, 2004). Studies revealed interactions between VirB6 and two outer membrane proteins, VirB7 and VirB9 (Jakubowski *et al.*, 2003). VirB6 through cooperation with VirB7/8/9/10 forms a portion of the protein translocation channel that directly interacts with the T-DNA/VirD2 complex (Jakubowski *et al.*, 2004). In fact VirB6 participates in the relay of T-DNA/VirD2 transfer from VirD4/VirB11/VirB4 subunits to VirB8 thus enabling the subsequent transfer of the nucleoprotein complex by VirB8 to VirB9 and VirB2 for translocation across the outer membrane (Jakubowski *et al.*, 2004).

### *T-pilus constituents and outer membrane accessory proteins*

VirB2 is the major pilin subunit and uses the Sec pathway for export across the inner membrane. Afterwards VirB2 undergoes a unique event that covalently links the amino acid residues present at the N- and C-terminal ends to create a closed circular protein (Lai *et al.*, 2002). Subcellular fractionation detected VirB2 and VirB5 in both the inner membrane/ periplasm and outer membrane fractions. In addition, these proteins were also found as extracellular products. VirB5 interacts with and stabilizes VirB2 and plays a role in biogenesis of the T-pilus (Krall *et al.*, 2002). VirB3 and VirB5 are required for substrate transfer; however, no protein-protein interactions are noted for VirB3 (Cascales and Christie, 2003). The T-pilus is thought to protrude through the protein channel and it is through the pilus that DNA passes from *A. tumefaciens* into susceptible plants (Cascales and Christie, 2004).

Protein VirB9 is predicted to contain nine β-strands that span the outer membrane and based on sequence analysis appears to be related to other known secretins (Christie, 2004). It is postulated to form the outer membrane secretion pore and to form intermolecular disulfide bonds with the lipoprotein, VirB7 (Spudich *et al.*, 1996) (Anderson *et al.*, 1996) (Baron *et al.*, 1997). Relevance of the presence of VirB9 in the outer membrane is suggested by evidence which shows contact between the T-DNA substrate and the VirB9 protein (Cascales and Christie, 2004), but passage of substrate through a pore formed by VirB9 has not yet been shown.

The working model has been that the pilus is the protein conduit through which type IV substrates exit the cell. Mutational analysis of various VirB components in *A. tumefaciens* revealed that substrate transfer can occur even in the absence of pilus. This lead Christie to suggest that a pilus-like organelle may not be a mandatory feature necessary to achieve type IV mediated secretion (Christie, 2004). On the other hand, novel extracellular appendages have been identified in pathogens that produce T4SS for effector molecule secretion. For example when *H. pylori* are grown on solid media it elaborates rigid needle-like projections that protrude from the poles (Rohde *et al.*, 2003). These are quite different from conjugative pili in that they are thicker with a diameter of 45 nm as opposed to 7 nm for the average conjugative pili (Yoshida *et al.*, 1998). The filaments made by *H. pylori* are unique because when cells are attached to host cells they are covered in a sheath made of VirB10-like proteins which are antigenically variable proteins (Rohde *et al.*, 2003). It was postulated that the sheath is changeable in order to evade the host immune response. Also *L. pneumophila* T4SS produces a morphologically unique surface structure that consist of a diffuse web of fibrous material around the cell composed of DotO and DotH effectors (Watarai *et al.*, 2001). This material does not resemble conjugative pili at all and is noted only before the bacterium bursts from the macrophage to spread to other cells (Watarai *et al.*, 2001). The role of the newly identified surface organelles in type IV secretion is unknown.

## Type I protein secretion

Several toxins and exoenzymes utilize the type I secretion system (T1SS) for extracellular transport. They include but are not limited to the *B. pertussis* adenylate cyclase toxin (Sory *et al.*, 1995), *E. coli* colicin (CvaC), leukotoxin (Ltka) of *Pseudomonas hemolytica*, and hemolysin (HlyA) produced by *E. coli*. The T1SS produced by *E. coli* has been studied extensively and will be used in this discussion as the model system. The translocase that transports HlyA consists of three proteins, the ATP binding cassette (ABC) transporter HlyB, the membrane fusion protein HlyD and the outer membrane component TolC (Andersen, 2003). The genes of the hemolysin operon are arranged as *hlyCABD*. The *tolC* gene is not linked to the *hlyA* operon but is part of the *mar-sox* regulon (Andersen, 2003).

Type I secretion systems are a significant group of structurally and functionally related complexes identified in almost all Gram-negative bacteria described to date. A major feature of proteins secreted via the T1SS is the presence of a variable number of glycine- and aspartic-rich repeats located at the C-terminal half of the secreted protein (Coote, 1992). This repeat in toxin (Coote, 1992) motif does not serve as the primary signal sequence. Instead, type I secreted proteins are

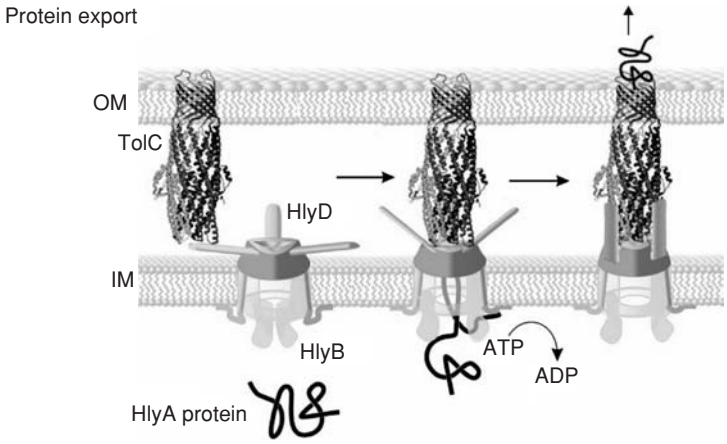



**FIGURE 5.4** Assembly of TolC-dependent export machinery. This model shows recruitment of TolC by the inner membrane translocase. Protein translocation occurs across the inner membrane (IM), outer membrane (OM), and the intervening periplasmic space. The accessory protein (Pimenta *et al.*, 1999) interacts with the ATP binding cassette (HlyB) transporter protein. Binding of substrate (HlyA) to the HlyD-HlyB subunit results in a conformational change in HlyD followed by recruitment of TolC. These interactions result in a tight trimolecular complex which spans the cell envelope. Simultaneously the protein translocation tunnel opens and substrate is secreted. Reprinted, with permission, from the *Annual Review of Biochemistry*, Volume 73 © 2004 by Annual Reviews www.annualreviews.org.

selected for entry into the pathway by recognition of a signal sequence located within the last 60 residues of the carboxy terminus. The recognition signal consists of three distinct features. Moving from the amino terminus is a 22 residue amphipathic α-helical domain, then next to that is a 13 amino acid uncharged sequence, followed by an eight residue hydrophobic region at the extreme carboxy terminus (Stanley *et al.*, 1991). Recognition of the signal sequence is believed to rely not only on the specific amino acid sequence but also on secondary structure (Stanley *et al.*, 1991) (Zhang *et al.*, 1993). The T1SS sorting sequence is not generally cleaved following translocation. The only known exception, to date, is the sorting sequence of the HasA protein, which is cleaved off during or after secretion to the extracellular environment (Izadi-Pruneyre *et al.*, 1999). The *E. coli* hemolysin T1SS has an additional feature in that a leader peptide located at the amino terminus and characterized by a double glycine motif is recognized by SecB and cleaved from the protein by the ABC transporter following translocation. This property is not a common attribute of other T1SS.

The hallmark of T1SS is that proteins are transported from the cytoplasm directly through the cell envelope to the extracellular environment without entering the periplasm (Koronakis *et al.*, 1989) (Andersen, 2003). Type I secretion occurs in distinct stages. First the accessory protein interacts with the ABC transporter. Then this heterodimer recognizes and binds to the signal recognition sequence of the protein to be secreted. This institutes a conformational change in the accessory protein allowing recruitment of TolC thus forming a tight trimolecular complex. Simultaneously, the protein translocation tunnel opens and the substrate is secreted. Following translocation of the protein TolC disassociates from the ABC transporter/accessory protein complex (Andersen, 2003) (Koronakis *et al.*, 2004).

Since the type I secretion substrates do not enter the periplasm, they have no access to disulfide bond (Dsb) forming enzymes commonly found in the periplasm or bound to the inner membrane facing the periplasm. Nonetheless some RTX toxins contain cysteine residues and lack of access of type I dependent proteins to Dsb enzymes do not affect their activity. For instance, colicin V was shown to contain disulfide bonds post secretion (Andersen, 2003). However, where and when disulfide bonds are formed is not known. Few proteins form stable disulfide bonds in the cytoplasm, and cytoplasmic enzymes such as thioredoxins (trxA, trxC), glutaredoxins (grxA, grxB, grxC) and glutathione, keep cysteine-containing proteins in their reduced state while in the cytoplasm (Zheng *et al.*, 1998).

### *ABC transporter*

The ABC transporter is a member of one of the largest protein superfamilies. There are 80 ABC transporters identified in *E. coli*, which are subdivided based on their substrate specificity and whether or not they participate in efflux or influx of materials. The type I specific ABC transporter assembles in the inner membrane as a homodimer. Self association results in four protein domains that consist of 2 hydrophobic membrane- spanning domains (MSD) and 2 cytoplasmic nucleotide-binding domains (NBD) (Saurin *et al.*, 1999). The NBD contains conserved sequences like the Walker A and Walker B motifs found in other ATPases. There is also a distinctive and conserved LSGGQ motif (linker peptide) present in almost all ABC transporters identified to date, that also participates in nucleotide binding (Hung *et al.*, 1998). Loss of function mutations in one nucleotide binding site results in no ATPase activity (Davidson and Chen, 2004) suggesting a level of cooperativity between the two subunits. Residues from both monomers are required to form the nucleotide binding site (Jones and George, 1999). X-ray crystallography of ABC transporters

reveals that ATP binds to a site that consists of residues in the Walker A box of one subunit and LSGGQ linker residues in the other subunit. It is still debated whether it is ATP binding or hydrolysis coupled to conformational changes in the membrane- spanning domains of the ABC transporter that mediates unidirectional transport of substrate (Davidson and Chen, 2004). Indeed whether both nucleotide-binding sites must be occupied before the ATPase becomes catalytically active (i.e. capable of transporting substrate) or whether ATP binding at one site promotes hydrolysis at the other is unclear (Davidson and Chen, 2004).

***The accessory protein***

The role of the accessory protein is to link the inner membrane ABC transporter to the outer membrane translocon and to stabilize the tripartite assembly during protein translocation across the outer membrane. The accessory protein is a bitopic protein with a large periplasmic domain and a short cytoplasmic domain anchored by a membrane- spanning region (Pimenta *et al.*, 1999) (Balakrishnan *et al.*, 2001). The short cytoplasmic domain has been implicated in substrate binding. The accessory protein forms homotrimers and is known to form a stable complex in the inner membrane with the ABC transporter. In fact, the ABC transporter is unstable in the absence of the accessory protein (Thanabalu *et al.*, 1998) (Pimenta *et al.*, 1999). Crosslinking studies reveal that prior to secretion type I dependent proteins independently bind both the accessory protein and the ABC transporter resulting in a conformational change that brings this complex into tight association with the outer membrane translocon, TolC (Thanabalu *et al.*, 1998) (Hwang *et al.*, 1997). Electrophysiological studies indicate that the open configuration of the translocation tunnel formed by TolC is stabilized in the presence of accessory protein.

***TolC: the protein translocation tunnel***

TolC is a multifunctional protein which has a role in hemolysin secretion, colicin import and multidrug efflux that removes toxic substances like antibiotics from the bacterial cell (Sulavik *et al.*, 2001). In fact one of the most important and effective multidrug efflux pumps produced by *E. coli*, AcrB, requires TolC (Fralick, 1996). Specifics of the mechanism of type I secretion was greatly enhanced after the X-ray crystallographic structure of TolC was solved to 2.1 Å resolution. Evidence indicates that monomers of TolC come together to form a loosely packed 12-stranded α/β barrel (Koronakis *et al.*, 2000). The TolC cylinder is comprised of 3 domains designated as the 40Å long channel region, 100Å tunnel domain, and the equatorial region (Koronakis *et al.*, 2000). The β-barrel channel is embedded in the outer membrane; the α-helical tunnel, which is an extension of the channel domain, spans the periplasm and is surrounded at the middle by a band of both α- and β-helices. The β-barrel channel portion of the cylinder is vastly different from other porins in that three TolC monomers together form one contiguous barrel whereas other known outer membrane porins normally form one β-barrel per monomer (Koronakis *et al.*, 2004). Moreover the large periplasmic α-helical barrel domain of TolC is distinctive in that the trimer forms a tapered cylinder measuring 140Å in length and is wide enough to transverse the periplasmic space. The average accessible interior diameter of the TolC pore is 19.8Å across its entire length except at the end that faces the periplasm (3.9 Å). The TolC β-barrel portion of the channel-tunnel is always open to the external environment. By contrast, the tapered arrangement of the α-helical coiled-coils at the bottom of the tunnel domain effectively seals the periplasmic entrance against passage of large molecules out of the cell, and the entire barrel follows a left-handed superhelical twist (Koronakis *et al.*, 2000) (Andersen *et al.*, 2002a) (Koronakis *et al.*, 2004).

A circular network of intra- and intermolecular bonding between six conserved aspartate residues and other charged amino acid residues located at the bottom of the tunnel keep the coiled coil packing configuration intact pulling the three inner coils close together to create the closed conformation. Site-directed mutagenesis and electrophysiological studies implicated residues D371 and D 374 located at consecutive helical turns in each of the three TolC monomers as crucial for maintenance of the closed state (Andersen *et al.*, 2002a). This electronegative inner surface is highly conserved in all TolC homologues (Koronakis *et al.*, 2000). A conformational change would be required to open the pore (Koronakis *et al.*, 2000). Mutational analyses indicate that a slight twisting of the α-helices of the three inner coiled coils realigns them with the outer coiled coil so that the entrance widens enough to allow entry of large molecules (Koronakis *et al.*, 2000) (Andersen *et al.*, 2002a) (Andersen *et al.*, 2002b) (Eswaran *et al.*, 2003). In fact, locked-mutants (inner coiled coils are no longer flexible) still bind hemolysin substrate to the HlyB/HlyD translocase and recruit TolC. Yet hemolysin is not secreted in the locked mutants suggesting that untwisting of the inner coil is required to open the gateway for substrate secretion (Eswaran *et al.*, 2003).

## The type III secretion pathway

Secretion via the type III secretion system (T3SS) occurs, just like the T1SS, in one step and completely

bypasses the periplasm. The T3SS is one of the most complex schemes identified to date for translocation of toxins across the bacterial outer membrane. This is a mechanism used by bacteria to transport proteins from the bacterial cytosol and following contact with host cells deliver these toxic molecules directly into the eukaryotic cell. Gram-negative bacteria pathogenic for human, animals and plants possess and utilize T3SSs. Indeed it is through the use of specialized effector proteins secreted by T3SS that some highly pathogenic organisms establish their niche. For example, *Yersinia pestis* injects effectors that target macrophages and allow this bacterium to evade phagcocytosis and survive the primary immune response of the host while it grows unfettered in lymphoid tissue. The hallmarks of disease of frank pathogens like *Salmonella typhi*, *Shigella flexneri, Y. pestis*, and enteropathogenic *E. coli* (EPEC) and plant pathogens like *Pseudomonas syringae, Erwinia* spp., and *Xanthomonas campestris* can be traced to type III dependent-effector molecules. Even opportunistic *P. aeruginosa* exploits this secretion modality with devastating consequences to patients with preexisting conditions such as extensive burns and cystic fibrosis.

Three *Yersinia* species are pathogenic for humans and rodents. Like the infamous agent of bubonic plague, *Y. pestis, Y. pseudotuberculosis*, and *Y. enterocolitica* have a marked tropism for lymphatic tissue, and all three species are capable of evading the host primary immune response and avoiding phagocytosis (Hueck, 1998). The type III pathway was first discovered in *Yersinia* (Rosqvist *et al.*, 1994) and has since been studied extensively. Therefore the *Yersinia* system will be used as the model for discussion and comparisons will be made where appropriate. In the case of *Yersinia* spp. and *S. flexneri* the T3SS genes are clustered together on large virulence plasmids whereas the genes carried by *Salmonella* spp. and EPEC are on the chromosome grouped within pathogenicity islands (Hueck, 1998). The overall genetic organization of type III gene clusters is conserved only within the different subgroups (Hueck, 1998) and there is no standard nomenclature used to identify these loci across species. Specifically, *S. typhimurium* carry two T3SSs called SPI-1 and SPI-2, which contribute to pathogenesis at different stages of infection. The SPI-1 genes are identified as *inv, spa, prg*, and *org*, and the most recently discovered SPI-2 genes are called *ssa* for secretion system apparatus. *S. flexneri* genes are designated as *mxi* and *spa* (Hueck, 1998).

### *Genes of the Yersinia T3SS*

The Yop proteins are split into two groups (Hueck, 1998). The first group includes the secreted effector proteins: YopE, YopH, YopM, YopO/YpkA, YopP/YopJ, and YopT. The second group is composed of secreted proteins that form the translocation pore in the plasma membrane of the host cell to allow delivery of toxins. They include– YopB, YopD, and LcrV. The Yersinia secretion machinery is encoded by four loci: *virC* (*yscA–M*), *virG* (*yscW*), *virB* (*yscN–U*) and *virA* (*yopN, tyeA, yscV* [formerly *lcrD*]) and *lcrR*. The *yop, syc*, and *ysc* genes are located on the 70 kb plasmid called pYV.

Six Yop chaperones (Iriarte and Cornelis, 1999) are each known to stabilize and support secretion of specific Yop proteins. They are designated as SycE (i.e. serves YopE), SycH (Sory *et al.*, 1995), SycC (formerly LcrH and serves both YopD and YopB), and SycT (YopT) (Hueck, 1998). YscB behaves as a chaperone for YopN and is expressly required for its normal secretion (Jackson *et al.*, 1998). YopN is not an effector protein but is rather a secreted factor that controls the release of other Yop proteins. Together YopN, LcrG and Tye may form a stop valve that regulates the opening and closing of the translocation pore. YscY is the chaperone of YscX the function of which is still under investigation (Iriarte and Cornelis, 1999).

### *Events that precede protein translocation.*

Upon entering the host, the temperature shift from ambient to 37˚C results in a change in the bending of the large virulence plasmid DNA harbored by *Yersinia* so that the promoters of type III secretion genes are more accessible to the positive regulator, VirF (Lambert de *et al.*, 1992) (Rohde *et al.*, 1999). This results in the expression of both the *ysc* and *yop* genes. However, an increase in temperature is not sufficient to induce Yop secretion. Other environmental signals such as serum albumin, specific amino acids, and low calcium ion concentration may be required to trigger substrate translocation (Lee *et al.*, 2001). Historical evidence shows that Yersinia grown at 26˚C in rich medium with calcium then shifted to 37˚C with calcium ions depleted resulted in Yop secretion (Michiels *et al.*, 1990) (Straley *et al.*, 1993). The physiological significance of calcium ion depletion for triggering Yop translocation is not readily apparent because *Yersinia* remains extracellular during infection in a calcium rich environment (Cornelis, 2003). On the other hand strong experimental evidence indicates that it is intimate contact of bacterial cells with the target cell that activates translocation of proteins into the eukaryotic cell (Pettersson *et al.*, 1996).

### *Chaperone and Yop targeting to the type III secretion machine*

The synthesis of Yops is believed to be controlled by a feedback inhibition mechanism and to involve both

Yops and specific chaperones (Cornelis *et al.*, 1987) (Francis *et al.*, 2002). Although the mechanisms involved in the feedback process are not well understood experiments showed that the concentration of specific chaperones is directly linked to the rate of secretion of the Yop that they control (Darwin and Miller, 2001). The Yop proteins do not contain a classical signal peptide (Michiels *et al.*, 1990) but they do possess a recognizable sorting signal that allows the T3SS to discriminate between its substrates and other proteins (Hueck, 1998) (Cornelis and Van, 2000) (Galan, 2001). There are two different models proposed for targeting substrates to the type III machinery. One model suggests that ~20 residues contained at the amino terminus are important for translocation (Sory *et al.*, 1995) (Anderson and Schneewind, 1997). Whether or not this signal is defined by the polypeptide sequence or the mRNA structure has been a matter of some debate since there is some evidence that supports both conclusions (Lloyd *et al.*, 2001) (Anderson and Schneewind, 1997) (Anderson and Schneewind, 1999).

The second model relies on the presence of a group of specific chaperones that bind to distinct domains on the proteins to be secreted to maintain them in a state fit for translocation (Wattiau *et al.*, 1996). Inactivation of chaperones results in loss of secretion and unstable effector proteins that are poorly expressed. There is also some suggestion that binding of chaperones results in the formation of a three-dimensional sorting signal important for uptake and transport through the type III pathway (Birtalan *et al.*, 2002). Most chaperones are acidic cytosolic proteins between 15–20 kDa. This group of proteins does not share amino acid sequence homology although they all have a common putative amphiphilic alpha-helix at their carboxy-terminus (Wattiau *et al.*, 1994). Comparison of the crystal structures of effector/chaperone complexes, SycE-YopE and SptP-SicP, of *Yersinia* and *Salmonella* respectively, indicates remarkable stereochemical conservation (Birtalan *et al.*, 2002) (Stebbins and Galan, 2001). The similarity may be indicative of a generalized mode of substrate-chaperone interaction and recognition. Work by Stebbins and Galán provide evidence that chaperones prevent substrates from pre-mature folding prior to translocation (Stebbins and Galan, 2001). Conversely YopE bound to SycE was shown to be active which suggests that it was already folded prior to translocation (Birtalan *et al.*, 2002). This clearly illustrates that there is much work to be done to learn the exact mechanism and function of the chaperones and to discern whether or not molecules fold before they enter the translocon.

Several laboratories have focused on defining the domains of type III substrates crucial for translocation. For instance, the SopE catalytic domain mapped to residues 78-240 (Buchwald *et al.*, 2002) and the first ~100 amino acids of SopE was sufficient to target a heterologous protein for secretion through the type III machinery (Evans *et al.*, 2003). Removal of the chaperone binding domain of both SptP and SopE made them incompetent for secretion by their specific type III systems (Lee and Galan, 2004) and exposed a cryptic flagella-uptake signal that shunted both SptP and SopE into the flagella export pathway. Thus the authors concluded that chaperones may endow their cognate substrates with type III secretion specificity (Lee and Galan, 2004).

### *Supramolecular protein secretion apparatus*

Several genes that encode components of the basal body assembly of flagellum share homology with those of the type III machinery which indicates that the two systems have a common evolutionary origin (Hueck, 1998). The type III secretion machine is highly conserved among the pathogens that possess them. Indeed electronmicrographs of the supramolecular structure of type III secretion machineries of *S. flexneri* and *S. typhimurium* reveal remarkable similarities between the two translocons (Kubori *et al.*, 1998) (Blocker *et al.*, 2001). Furthermore electron microscopy of the protein conduits of *S. flexneri*, *S. typhimurium*, and *Y. entercolitica* indicate that these three organisms fashion similar needle-like hydrophobic projections that cross or may pierce the eukaryotic plasma membrane (Blocker *et al.*, 2001) (Kubori *et al.*, 2000) (Hoiczyk and Blobel, 2001). Further evidence suggests that the secretion machines are interchangeable. For instance the *Y. pseudotuberculosis* type III apparatus recognized and translocated *P. aeruginosa* exoenzyme S toxin (Errington *et al.*, 2003), a type III substrate, into HeLa cells (Frithz-Lindsten *et al.*, 1997).

Electron microscopy of *S. typhimurium* revealed that the type III secretion structure has a base which begins at the bacterial inner membrane and extends to the outer membrane where it connects with a needle-like projection that protrudes from the bacterial surface. Complexes extracted from the membrane have a cylindrical symmetry. Moreover, the base structure contains two upper and two lower rings which anchor the needle-like projection to the cell envelope. The needle consists of a stiff straight tube, 80 nm long and 13 nm wide, and appears hollow (Kubori *et al.*, 1998). In addition to the needle complex all T3SSs have a group of highly conserved integral membrane proteins located at the base of the needle complex to facilitate passage of the secreted proteins through the bacterial inner membrane (Cornelis and Van, 2000).

Like the basal body assembly component of flagella, there is an ATPase associated with the needle complex

of the type III apparatus thought to drive secreted proteins through the machinery (Hueck, 1998) (Cornelis and Van, 2000). The catalytic β subunit of the flagella ATPase and the type III ATPase display a high level of amino acid sequence similarity. Recently type III-associated ATPase was found to be crucial for functionality of the type III machinery (Akeda and Galan, 2004). Three distinct domains of the protein were identified. The N-terminal end of the ATPase participates in membrane association, while the central portion of the protein is important for self-association. Finally the C-terminal domain contains the catalytic ATPase site.

The length and width of the T3SS needle complex varies among bacteria that produce it. For example in EPEC the needle extends between 75-260 nm with a diameter of 10-12 nm (Daniell *et al.*, 2001). In *Yersinia* the YscP protein appears to act as a molecular ruler to control the length of the needle that consist of polymerized YscF (Journet *et al.*, 2003). Amazingly, the length of the needle is dependent on the number of amino acid residues present within the regulatory protein YscP. It is believed that YscP positions itself by anchoring to the basal body with its other end attached to the growing tip of the needle and when the needle reaches the length of the regulator protein polymerization of YscF stops.

### Asymmetric localization of secretory machineries

There are many examples of individual proteins and large supramolecular complexes restricted to discrete locations within the cell. For instance, chemoreceptors of *Caulobacter* are distributed to the cell pole while proteins involved in cell division are restricted to the septum (Alley *et al.*, 1992) (Buddelmeijer and Beckwith, 2002) (Errington *et al.*, 2003). Recently it was revealed that the Sec machinery produced by *Streptococcus pyogenes* localize to discrete foci distal from the new and old poles (Rosch and Caparon, 2004). Similarly the type II apparatus of *Vibrio cholerae* is localized to the old pole of the cell (Scott *et al.*, 2001). The mechanism of restrictive positioning of the type II apparatus is unknown but preliminary data indicate that the M protein (EpsM) may play an important role. EpsM is distributed to the pole when expressed in *E. coli* in the absence of other type II proteins, unlike EpsL which disperses throughout the cell membrane. Yet, co-expression of EpsM with EpsL results in polar localization of EpsL (Scott *et al.*, 2001). Therefore EpsM may serve as nucleation factor for polar assembly of the *V. cholerae* type II secretion complex or a subcomplex thereof. Localization of the type II apparatus at the pole of the cell may be an advantageous method of concentrating the relatively low number of secretion complexes as a means to deliver secreted material exactly where it is needed without waste or dilution.

The coupling protein of the type IV secretion machinery produced by *A. tumefaciens* was also determined to localize at the pole of the cell (Kumar and Das, 2002). This data supports an earlier observation that *Agrobacterium* attaches to the plant cell in a polar manner (Llosa and O'Callaghan, 2004). It is not known whether other secretion machineries such as type I and III are maintained at the pole or localized to some discrete domain of the cell envelope but theoretically, directed secretion may offer the same possible survival advantage to other species of bacteria as well. For example the type III secretion apparatus has only been viewed following *in vitro* induction of secretion genes. Under these conditions the needle-like projections that form the type III apparatus appear to be indiscriminately placed along the periphery of the cell membrane. This observation begs the question of whether or not these machines would aggregate to one discrete site when legitimate induction occurs.

## CLOSING REMARKS

Bacterial protein secretion is an exciting and expanding field. Scrutiny of the current systems reveals basic repetitive themes used to translocate proteins from the cytoplasm and across lipid barriers. Pathogenic bacteria have devised systems to deliver poisons directly to their host and in some cases in expeditious and thrifty ways. A perfect example is autotransporters that exploit the Sec pathway and achieve translocation across the outer membrane without a multimolecular secretion apparatus. Yet equally fascinating is the type III system which has usurped the perfected design of the basal body assembly of flagella and turned it with a few important changes into an efficient delivery machine. Let's not forget the asymmetrically positioned type II and type IV systems that harbor ancestral genes to create vessels that span the cell envelope to carry effectors to the host. It is possible that additional and perhaps mysterious secretion systems are yet to be discovered. As each new and old system is studied, weaknesses are revealed which may become therapeutic targets for the new generation of antibiotic drugs.

## REFERENCES

Akeda, Y. and Galan, J.E. (2004). Genetic analysis of the Salmonella enterica type III secretion-associated ATPase InvC defines discrete functional domains. J. Bacteriol. **186**, 2402–2412.

Akrim, M., Bally, M., Ball, G., Tommassen, J., Teerink, H., Filloux, A., and Lazdunski, A. (1993). Xcp-mediated protein secretion in Pseudomonas aeruginosa: identification of two additional genes and evidence for regulation of xcp gene expression. Mol. Microbiol. **10**, 431–443.

Alley, M.R., Maddock, J.R., and Shapiro, L. (1992). Polar localization of a bacterial chemoreceptor. Genes Dev. **6**, 825–836.

Andersen, C. (2003). Channel-tunnels: outer membrane components of type I secretion systems and multidrug efflux pumps of Gram-negative bacteria. Rev. Physiol Biochem. Pharmacol. **147**, 122–165.

Andersen, C., Koronakis, E., Bokma, E., Eswaran, J., Humphreys, D., Hughes, C., and Koronakis, V. (2002a). Transition to the open state of the TolC periplasmic tunnel entrance. Proc. Natl. Acad. Sci. U. S. A **99**, 11103–11108.

Andersen, C., Koronakis, E., Hughes, C., and Koronakis, V. (2002b). An aspartate ring at the TolC tunnel entrance determines ion selectivity and presents a target for blocking by large cations. Mol. Microbiol. **44**, 1131–1139.

Anderson, D.M. and Schneewind, O. (1999). Yersinia enterocolitica type III secretion: an mRNA signal that couples translation and secretion of YopQ. Mol. Microbiol. **31**, 1139–1148.

Anderson, D.M. and Schneewind, O. (1997). A mRNA signal for the type III secretion of Yop proteins by Yersinia enterocolitica. Science **278**, 1140–1143.

Anderson, L.B., Hertzel, A.V., and Das, A. (1996). Agrobacterium tumefaciens VirB7 and VirB9 form a disulfide-linked protein complex. Proc. Natl. Acad. Sci. U. S. A **93**, 8889–8894.

Atmakuri, K., Cascales, E., and Christie, P.J. (2004). Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. Mol. Microbiol. **54**, 1199–1211.

Atmakuri, K., Ding, Z., and Christie, P.J. (2003). VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of Agrobacterium tumefaciens. Mol. Microbiol. **49**, 1699–1713.

Bachman, M.A. and Swanson, M.S. (2001). RpoS co-operates with other factors to induce Legionella pneumophila virulence in the stationary phase. Mol. Microbiol. **40**, 1201–1214.

Balakrishnan, L., Hughes, C., and Koronakis, V. (2001). Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by Escherichia coli. J. Mol. Biol. **313**, 501–510.

Ball, G., Chapon-Herve, V., Bleves, S., Michel, G., and Bally, M. (1999). Assembly of XcpR in the cytoplasmic membrane is required for extracellular protein secretion in Pseudomonas aeruginosa. J. Bacteriol. **181**, 382–388.

Baron, C., Thorstenson, Y.R., and Zambryski, P.C. (1997). The lipoprotein VirB7 interacts with VirB9 in the membranes of Agrobacterium tumefaciens. J. Bacteriol. **179**, 1211–1218.

Berks, B.C. (1996). A common export pathway for proteins binding complex redox cofactors? Mol. Microbiol. **22**, 393–404.

Berks, B.C., Sargent, F., De, L.E., Hinsley, A.P., Stanley, N.R., Jack, R.L., Buchanan, G., and Palmer, T. (2000a). A novel protein transport system involved in the biogenesis of bacterial electron transfer chains. Biochim. Biophys. Acta **1459**, 325–330.

Berks, B.C., Sargent, F., and Palmer, T. (2000b). The Tat protein export pathway. Mol. Microbiol. **35**, 260–274.

Birtalan, S.C., Phillips, R.M., and Ghosh, P. (2002). Three-dimensional secretion signals in chaperone-effector complexes of bacterial pathogens. Mol. Cell **9**, 971–980.

Bitter, W., Koster, M., Latijnhouwers, M., de, C.H., and Tommassen, J. (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of Pseudomonas aeruginosa. Mol. Microbiol. **27**, 209–219.

Blaudeck, N., Sprenger, G.A., Freudl, R., and Wiegert, T. (2001). Specificity of signal peptide recognition in tat-dependent bacterial protein translocation. J. Bacteriol. **183**, 604–610.

Bleves, S., Voulhoux, R., Michel, G., Lazdunski, A., Tommassen, J., and Filloux, A. (1998). The secretion apparatus of Pseudomonas aeruginosa: identification of a fifth pseudopilin, XcpX (GspK family). Mol. Microbiol. **27**, 31–40.

Blocker, A., Jouihri, N., Larquet, E., Gounon, P., Ebel, F., Parsot, C., Sansonetti, P., and Allaoui, A. (2001). Structure and composition of the Shigella flexneri "needle complex", a part of its type III secreton. Mol. Microbiol. **39**, 652–663.

Bogsch, E.G., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C., and Palmer, T. (1998). An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. J. Biol. Chem. **273**, 18003–18006.

Bolhuis, A., Mathers, J.E., Thomas, J.D., Barrett, C.M., and Robinson, C. (2001). TatB and TatC form a functional and structural unit of the twin-arginine translocase from Escherichia coli. J. Biol. Chem. **276**, 20213–20219.

Bortoli-German, I., Brun, E., Py, B., Chippaux, M., and Barras, F. (1994). Periplasmic disulphide bond formation is essential for cellulase secretion by the plant pathogen Erwinia chrysanthemi. Mol. Microbiol. **11**, 545–553.

Braun, P., Tommassen, J., and Filloux, A. (1996). Role of the propeptide in folding and secretion of elastase of Pseudomonas aeruginosa. Mol. Microbiol. **19**, 297–306.

Brok, R., Van, G.P., Winterhalter, M., Ziese, U., Koster, A.J., de, C.H., Koster, M., Tommassen, J., and Bitter, W. (1999). The C-terminal domain of the Pseudomonas secretin XcpQ forms oligomeric rings with pore activity. J. Mol. Biol. **294**, 1169–1179.

Buchwald, G., Friebel, A., Galan, J.E., Hardt, W.D., Wittinghofer, A., and Scheffzek, K. (2002). Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE. EMBO J. **21**, 3286–3295.

Buddelmeijer, N. and Beckwith, J. (2002). Assembly of cell division proteins at the E. coli cell center. Curr. Opin. Microbiol. **5**, 553–557.

Burns, D.L. (2003). Type IV transporters of pathogenic bacteria. Curr. Opin. Microbiol. **6**, 29–34.

Camberg, J.L. and Sandkvist, M. (2005). Molecular Analysis of the Vibrio cholerae Type II Secretion ATPase EpsE. J. Bacteriol. **187**, 249–256.

Cascales, E. and Christie, P.J. (2003). The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. **1**, 137–149.

Cascales, E. and Christie, P.J. (2004). Definition of a bacterial type IV secretion pathway for a DNA substrate. Science **304**, 1170–1173.

Chang, C.H. and Winans, S.C. (1992). Functional roles assigned to the periplasmic, linker, and receiver domains of the Agrobacterium tumefaciens VirA protein. J. Bacteriol. **174**, 7033–7039.

Chapon, V., Czjzek, M., El, H.M., Py, B., Juy, M., and Barras, F. (2001). Type II protein secretion in gram-negative pathogenic bacteria: the study of the structure/secretion relationships of the cellulase Cel5 (formerly EGZ) from Erwinia chrysanthemi. J. Mol. Biol. **310**, 1055–1066.

Christie, P.J. (2004). Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems. Biochim. Biophys. Acta **1694**, 219–234.

Clemons, W.M., Jr., Menetret, J.F., Akey, C.W., and Rapoport, T.A. (2004). Structural insight into the protein translocation channel. Curr. Opin. Struct. Biol. **14**, 390–396.

Cline, K. and Mori, H. (2001). Thylakoid DeltapH-dependent precursor proteins bind to a cpTatC-Hcf106 complex before Tha4–dependent transport. J. Cell Biol. **154**, 719–729.

Collier, D.N., Bankaitis, V.A., Weiss, J.B., and Bassford, P.J., Jr. (1988). The antifolding activity of SecB promotes the export of the E. coli maltose-binding protein. Cell **53**, 273–283.

Connell, T.D., Metzger, D.J., Wang, M., Jobling, M.G., and Holmes, R.K. (1995). Initial studies of the structural signal for extracellular transport of cholera toxin and other proteins recognized by Vibrio cholerae. Infect. Immun. **63**, 4091–4098.

Coote, J.G. (1992). Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. FEMS Microbiol. Rev. **8**, 137–161.

Cornelis, G., Vanootegem, J.C., and Sluiters, C. (1987). Transcription of the yop regulon from Y. enterocolitica requires trans acting pYV and chromosomal genes. Microb. Pathog. **2**, 367–379.

Cornelis, G.R. (2003). How Yops find their way out of Yersinia. Mol. Microbiol. **50**, 1091–1094.

Cornelis, G.R. and Van, G.F. (2000). Assembly and function of type III secretory systems. Annu. Rev. Microbiol. **54**, 735–774.

d'Enfert, C. and Pugsley, A.P. (1989). Klebsiella pneumoniae pulS gene encodes an outer membrane lipoprotein required for pullulanase secretion. J. Bacteriol. **171**, 3673–3679.

d'Enfert, C., Ryter, A., and Pugsley, A.P. (1987). Cloning and expression in Escherichia coli of the Klebsiella pneumoniae genes for production, surface localization and secretion of the lipoprotein pullulanase. EMBO J. **6**, 3531–3538.

Dalbey, R.E. and Kuhn, A. (2000). Evolutionarily related insertion pathways of bacterial, mitochondrial, and thylakoid membrane proteins. Annu. Rev. Cell Dev. Biol. **16**, 51–87.

Dalbey, R.E., Lively, M.O., Bron, S., and van Dijl, J.M. (1997). The chemistry and enzymology of the type I signal peptidases. Protein Sci. **6**, 1129–1138.

Dang, T.A. and Christie, P.J. (1997). The VirB4 ATPase of Agrobacterium tumefaciens is a cytoplasmic membrane protein exposed at the periplasmic surface. J. Bacteriol. **179**, 453–462.

Daniell, S.J., Takahashi, N., Wilson, R., Friedberg, D., Rosenshine, I., Booy, F.P., Shaw, R.K., Knutton, S., Frankel, G., and Aizawa, S. (2001). The filamentous type III secretion translocon of enteropathogenic Escherichia coli. Cell Microbiol. **3**, 865–871.

Darwin, A.J. and Miller, V.L. (2001). The psp locus of Yersinia enterocolitica is required for virulence and for growth in vitro when the Ysc type III secretion system is produced. Mol. Microbiol. **39**, 429–444.

Davidson, A.L. and Chen, J. (2004). ATP-binding cassette transporters in bacteria. Annu. Rev. Biochem. **73**, 241–268.

de Gier, J.W. and Luirink, J. (2003). The ribosome and YidC. New insights into the biogenesis of Escherichia coli inner membrane proteins. EMBO Rep. **4**, 939–943.

de, G.A., Filloux, A., and Tommassen, J. (1991). Conservation of xcp genes, involved in the two-step protein secretion process, in different Pseudomonas species and other gram-negative bacteria. Mol. Gen. Genet. **229**, 278–284.

de, G.A., Koster, M., Gerard-Vincent, M., Gerritse, G., Lazdunski, A., Tommassen, J., and Filloux, A. (2001). Exchange of Xcp (Gsp) secretion machineries between Pseudomonas aeruginosa and Pseudomonas alcaligenes: species specificity unrelated to substrate recognition. J. Bacteriol. **183**, 959–967.

De, L.E., Porcelli, I., Sargent, F., Palmer, T., and Berks, B.C. (2001). Membrane interactions and self-association of the TatA and TatB components of the twin-arginine translocation pathway. FEBS Lett. **506**, 143–148.

DeLisa, M.P., Tullman, D., and Georgiou, G. (2003). Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. Proc. Natl. Acad. Sci. U. S. A **100**, 6115–6120.

Desvaux, M., Parham, N.J., and Henderson, I.R. (2004a). The autotransporter secretion system. Res. Microbiol. **155**, 53–60.

Desvaux, M., Parham, N.J., Scott-Tucker, A., and Henderson, I.R. (2004b). The general secretory pathway: a general misnomer? Trends Microbiol. **12**, 306–309.

Dillard, J.P. and Seifert, H.S. (2001). A variable genetic island specific for Neisseria gonorrhoeae is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. Mol. Microbiol. **41**, 263–277.

Douet, V., Loiseau, L., Barras, F., and Py, B. (2004). Systematic analysis, by the yeast two-hybrid, of protein interaction between components of the type II secretory machinery of Erwinia chrysanthemi. Res. Microbiol. **155**, 71–75.

Dreusch, A., Burgisser, D.M., Heizmann, C.W., and Zumft, W.G. (1997). Lack of copper insertion into unprocessed cytoplasmic nitrous oxide reductase generated by an R20D substitution in the arginine consensus motif of the signal peptide. Biochim. Biophys. Acta **1319**, 311–318.

Drew, D., Sjostrand, D., Nilsson, J., Urbig, T., Chin, C.N., de Gier, J.W., and von, H.G. (2002). Rapid topology mapping of Escherichia coli inner-membrane proteins by prediction and PhoA/GFP fusion analysis. Proc. Natl. Acad. Sci. U. S. A **99**, 2690–2695.

Duong, F. and Wickner, W. (1997). The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. EMBO J. **16**, 4871–4879.

Durand, E., Bernadac, A., Ball, G., Lazdunski, A., Sturgis, J.N., and Filloux, A. (2003). Type II protein secretion in Pseudomonas aeruginosa: the pseudopilus is a multifibrillar and adhesive structure. J. Bacteriol. **185**, 2749–2758.

El, K.M., Ockhuijsen, C., Bitter, W., Jaeger, K.E., and Tommassen, J. (1999). Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor. Mol. Gen. Genet. **261**, 770–776.

Errington, J., Daniel, R.A., and Scheffers, D.J. (2003). Cytokinesis in bacteria. Microbiol. Mol. Biol. Rev. **67**, 52–65, table.

Eswaran, J., Hughes, C., and Koronakis, V. (2003). Locking TolC entrance helices to prevent protein translocation by the bacterial type I export apparatus. J. Mol. Biol. **327**, 309–315.

Evans, D.T. **et al.** (2003). Mucosal priming of simian immunodeficiency virus-specific cytotoxic T-lymphocyte responses in rhesus macaques by the Salmonella type III secretion antigen delivery system. J. Virol. **77**, 2400–2409.

Fekkes, P., van der, D.C., and Driessen, A.J. (1997). The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. EMBO J. **16**, 6105–6113.

Filloux, A., Bally, M., Ball, G., Akrim, M., Tommassen, J., and Lazdunski, A. (1990). Protein secretion in gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. EMBO J. **9**, 4323–4329.

Findlay, G., Yu, J., and Hirst, T.R. (1993). Analysis of enterotoxin synthesis in a Vibrio cholerae strain lacking DsbA, a periplasmic enzyme involved in disulphide bond formation. Biochem. Soc. Trans. **21**, 212S.

Fralick, J.A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of Escherichia coli. J. Bacteriol. **178**, 5803–5805.

Francis, M.S., Wolf-Watz, H., and Forsberg, A. (2002). Regulation of type III secretion systems. Curr. Opin. Microbiol. **5**, 166–172.

Frithz-Lindsten, E., Du, Y., Rosqvist, R., and Forsberg, A. (1997). Intracellular targeting of exoenzyme S of Pseudomonas aeruginosa via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. Mol. Microbiol. **25**, 1125–1139.

Galan, J.E. (2001). Salmonella interactions with host cells: type III secretion at work. Annu. Rev. Cell Dev. Biol. **17**, 53–86.

Goldberg, M.B. and Sansonetti, P.J. (1993). Shigella subversion of the cellular cytoskeleton: a strategy for epithelial colonization. Infect. Immun. **61**, 4941–4946.

Gomis-Ruth, F.X. and Coll, M. (2001). Structure of TrwB, a gatekeeper in bacterial conjugation. Int. J. Biochem. Cell Biol. **33**, 839–843.

Gomis-Ruth, F.X., Moncalian, G., de la, C.F., and Coll, M. (2002). Conjugative plasmid protein TrwB, an integral membrane type IV secretion system coupling protein. Detailed structural features and mapping of the active site cleft. J. Biol. Chem. **277**, 7556–7566.

Gross, R., Simon, J., and Kroger, A. (1999). The role of the twin-arginine motif in the signal peptide encoded by the hydA gene of the hydrogenase from wolinella succinogenes. Arch. Microbiol. **172**, 227–232.

Halbig, D., Wiegert, T., Blaudeck, N., Freudl, R., and Sprenger, G.A. (1999). The efficient export of NADP-containing glucose-fructose oxidoreductase to the periplasm of Zymomonas mobilis depends both on an intact twin-arginine motif in the signal peptide and on the generation of a structural export signal induced by cofactor binding. Eur. J. Biochem. **263**, 543–551.

Hardie, K.R., Seydel, A., Guilvout, I., and Pugsley, A.P. (1996). The secretin-specific, chaperone-like protein of the general secretory pathway: separation of proteolytic protection and piloting functions. Mol. Microbiol. **22**, 967–976.

Hardy, S.J., Holmgren, J., Johansson, S., Sanchez, J., and Hirst, T.R. (1988). Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A **85**, 7109–7113.

Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P., and Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane. Cell **63**, 269–279.

Henderson, I.R. and Nataro, J.P. (2001). Virulence functions of autotransporter proteins. Infect. Immun. **69**, 1231–1243.

Henderson, I.R., Navarro-Garcia, F., and Nataro, J.P. (1998). The great escape: structure and function of the autotransporter proteins. Trends Microbiol. **6**, 370–378.

Hirst, T.R. and Holmgren, J. (1987b). Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from Vibrio cholerae. Proc. Natl. Acad. Sci. U. S. A **84**, 7418–7422.

Hirst, T.R. and Holmgren, J. (1987a). Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from Vibrio cholerae. J. Bacteriol. **169**, 1037–1045.

Hofreuter, D., Odenbreit, S., and Haas, R. (2001). Natural transformation competence in Helicobacter pylori is mediated by the basic components of a type IV secretion system. Mol. Microbiol. **41**, 379–391.

Hoiczyk, E. and Blobel, G. (2001). Polymerization of a single protein of the pathogen Yersinia enterocolitica into needles punctures eukaryotic cells. Proc. Natl. Acad. Sci. U. S. A **98**, 4669–4674.

Howard, S.P., Meiklejohn, H.G., Shivak, D., and Jahagirdar, R. (1996). A TonB-like protein and a novel membrane protein containing an ATP-binding cassette function together in exotoxin secretion. Mol. Microbiol. **22**, 595–604.

Hueck, C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62**, 379–433.

Hung, L.W., Wang, I.X., Nikaido, K., Liu, P.Q., Ames, G.F., and Kim, S.H. (1998). Crystal structure of the ATP-binding subunit of an ABC transporter. Nature **396**, 703–707.

Hunt, J.F., Weinkauf, S., Henry, L., Fak, J.J., McNicholas, P., Oliver, D.B., and Deisenhofer, J. (2002). Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. Science **297**, 2018–2026.

Hwang, J., Zhong, X., and Tai, P.C. (1997). Interactions of dedicated export membrane proteins of the colicin V secretion system: CvaA, a member of the membrane fusion protein family, interacts with CvaB and TolC. J. Bacteriol. **179**, 6264–6270.

Ignatova, Z., Hornle, C., Nurk, A., and Kasche, V. (2002). Unusual signal peptide directs penicillin amidase from Escherichia coli to the Tat translocation machinery. Biochem. Biophys. Res. Commun. **291**, 146–149.

Iriarte, M. and Cornelis, G.R. (1999). Identification of SycN, YscX, and YscY, three new elements of the Yersinia yop virulon. J. Bacteriol. **181**, 675–680.

Izadi-Pruneyre, N., Wolff, N., Redeker, V., Wandersman, C., Delepierre, M., and Lecroisey, A. (1999). NMR studies of the C-terminal secretion signal of the haem-binding protein, HasA. Eur. J. Biochem. **261**, 562–568.

Jack, R.L., Buchanan, G., Dubini, A., Hatzixanthis, K., Palmer, T., and Sargent, F. (2004). Coordinating assembly and export of complex bacterial proteins. EMBO J. **23**, 3962–3972.

Jack, R.L., Sargent, F., Berks, B.C., Sawers, G., and Palmer, T. (2001). Constitutive expression of Escherichia coli tat genes indicates an important role for the twin-arginine translocase during aerobic and anaerobic growth. J. Bacteriol. **183**, 1801–1804.

Jackson, M.W., Day, J.B., and Plano, G.V. (1998). YscB of Yersinia pestis functions as a specific chaperone for YopN. J. Bacteriol. **180**, 4912–4921.

Jacob-Dubuisson, F., Locht, C., and Antoine, R. (2001). Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. Mol. Microbiol. **40**, 306–313.

Jahagirdar, R. and Howard, S.P. (1994). Isolation and characterization of a second exe operon required for extracellular protein secretion in Aeromonas hydrophila. J. Bacteriol. **176**, 6819–6826.

Jakubowski, S.J., Krishnamoorthy, V., Cascales, E., and Christie, P.J. (2004). Agrobacterium tumefaciens VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion System. J. Mol. Biol. **341**, 961–977.

Jakubowski, S.J., Krishnamoorthy, V., and Christie, P.J. (2003). Agrobacterium tumefaciens VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. J. Bacteriol. **185**, 2867–2878.

Jones, P.M. and George, A.M. (1999). Subunit interactions in ABC transporters: towards a functional architecture. FEMS Microbiol. Lett. **179**, 187–202.

Journet, L., Agrain, C., Broz, P., and Cornelis, G.R. (2003). The needle length of bacterial injectisomes is determined by a molecular ruler. Science **302**, 1757–1760.

Kagami, Y., Ratliff, M., Surber, M., Martinez, A., and Nunn, D.N. (1998). Type II protein secretion by Pseudomonas aeruginosa: genetic suppression of a conditional mutation in the pilin-like component XcpT by the cytoplasmic component XcpR. Mol. Microbiol. **27**, 221–233.

Kihara, A., Akiyama, Y., and Ito, K. (1995). FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. Proc. Natl. Acad. Sci. U. S. A **92**, 4532–4536.

Koronakis, V., Eswaran, J., and Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. Annu. Rev. Biochem. **73**, 467–489.

Koronakis, V., Koronakis, E., and Hughes, C. (1989). Isolation and analysis of the C-terminal signal directing export of Escherichia coli hemolysin protein across both bacterial membranes. EMBO J. **8**, 595–605.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature **405**, 914–919.

Krall, L., Wiedemann, U., Unsin, G., Weiss, S., Domke, N., and Baron, C. (2002). Detergent extraction identifies different VirB protein subassemblies of the type IV secretion machinery in the membranes of Agrobacterium tumefaciens. Proc. Natl. Acad. Sci. U. S. A **99**, 11405–11410.

Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J.E., and Aizawa, S.I. (1998). Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science **280**, 602–605.

Kubori, T., Sukhan, A., Aizawa, S.I., and Galan, J.E. (2000). Molecular characterization and assembly of the needle complex of the Salmonella typhimurium type III protein secretion system. Proc. Natl. Acad. Sci. U. S. A **97**, 10225–10230.

Kumamoto, C.A. and Beckwith, J. (1983). Mutations in a new gene, secB, cause defective protein localization in Escherichia coli. J. Bacteriol. **154**, 253–260.

Kumar, R.B. and Das, A. (2002). Polar location and functional domains of the Agrobacterium tumefaciens DNA transfer protein VirD4. Mol. Microbiol. **43**, 1523–1532.

Lai, E.M., Eisenbrandt, R., Kalkum, M., Lanka, E., and Kado, C.I. (2002). Biogenesis of T pili in Agrobacterium tumefaciens requires precise VirB2 propilin cleavage and cyclization. J. Bacteriol. **184**, 327–330.

Lambert de, R.C., Sluiters, C., and Cornelis, G.R. (1992). Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of Yersinia enterocolitica. Mol. Microbiol. **6**, 395–409.

Lawley,T.D., Klimke, W.A., Gubbins, M.J., and Frost, L.S. (2003). F factor conjugation is a true type IV secretion system. FEMS Microbiol. Lett. **224**, 1–15.

Lee, S.H. and Galan, J.E. (2004). Salmonella type III secretion-associated chaperones confer secretion-pathway specificity. Mol. Microbiol. **51**, 483–495.

Lee, V.T., Mazmanian, S.K., and Schneewind, O. (2001). A program of Yersinia enterocolitica type III secretion reactions is activated by specific signals. J. Bacteriol. **183**, 4970–4978.

Linderoth, N.A., Simon, M.N., and Russel, M. (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. Science **278**, 1635–1638.

Llosa, M., Gomis-Ruth, F.X., Coll, M., and de la Cruz, F.F. (2002). Bacterial conjugation: a two-step mechanism for DNA transport. Mol. Microbiol. **45**, 1–8.

Llosa, M. and O'Callaghan, D. (2004). Euroconference on the Biology of Type IV Secretion Processes: bacterial gates into the outer world. Mol. Microbiol. **53**, 1–8.

Llosa, M., Zunzunegui, S., and de la, C.F. (2003). Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. Proc. Natl. Acad. Sci. U. S. A **100**, 10465–10470.

Lloyd, S.A., Norman, M., Rosqvist, R., and Wolf-Watz, H. (2001). Yersinia YopE is targeted for type III secretion by N-terminal, not mRNA, signals. Mol. Microbiol. **39**, 520–531.

Lu, H.M. and Lory, S. (1996). A specific targeting domain in mature exotoxin A is required for its extracellular secretion from Pseudomonas aeruginosa. EMBO J. **15**, 429–436.

Luirink, J. and Sinning, I. (2004). SRP-mediated protein targeting: structure and function revisited. Biochim. Biophys. Acta **1694**, 17–35.

Luo, Z.Q. and Isberg, R.R. (2004). Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. Proc. Natl. Acad. Sci. U. S. A **101**, 841–846.

Lupetti, P., Heuser, J.E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P.L., Dallai, R., Rappuoli, R., and Telford, J.L. (1996). Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. J. Cell Biol. **133**, 801–807.

McVay, C.S. and Hamood, A.N. (1995). Toxin A secretion in Pseudomonas aeruginosa: the role of the first 30 amino acids of the mature toxin. Mol. Gen. Genet. **249**, 515–525.

Melton, A.R. and Weiss, A.A. (1989). Environmental regulation of expression of virulence determinants in Bordetella pertussis. J. Bacteriol. **171**, 6206-6212.

Michel, G., Bleves, S., Ball, G., Lazdunski, A., and Filloux, A. (1998). Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in Pseudomonas aeruginosa. Microbiology **144 (Pt 12)**, 3379–3386.

Michiels,T., Wattiau, P., Brasseur, R., Ruysschaert, J.M., and Cornelis, G. (1990). Secretion of Yop proteins by Yersiniae. Infect. Immun. **58**, 2840–2849.

Nishiyama, K., Suzuki,T., and Tokuda, H. (1996). Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. Cell **85**, 71–81.

Nouwen, N., Stahlberg, H., Pugsley, A.P., and Engel, A. (2000). Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy. EMBO J. **19**, 2229–2236.

Nunn, D.N. and Lory, S. (1993). Cleavage, methylation, and localization of the Pseudomonas aeruginosa export proteins XcpT, -U, -V, and -W. J. Bacteriol. **175**, 4375–4382.

Ochsner, U.A., Snyder, A., Vasil, A.I., and Vasil, M.L. (2002). Effects of the twin-arginine translocase on secretion of virulence factors, stress response, and pathogenesis. Proc. Natl. Acad. Sci. U. S. A **99**, 8312–8317.

Ohnishi, Y., Nishiyama, M., Horinouchi, S., and Beppu,T. (1994). Involvement of the COOH-terminal pro-sequence of Serratia marcescens serine protease in the folding of the mature enzyme. J. Biol. Chem. **269**, 32800–32806.

Oliver, D.B. and Beckwith, J. (1981). E. coli mutant pleiotropically defective in the export of secreted proteins. Cell **25**, 765–772.

Oliver, D.C., Huang, G., and Fernandez, R.C. (2003a). Identification of secretion determinants of the Bordetella pertussis BrkA autotransporter. J. Bacteriol. **185**, 489–495.

Oliver, D.C., Huang, G., Nodel, E., Pleasance, S., and Fernandez, R.C. (2003b). A conserved region within the Bordetella pertussis autotransporter BrkA is necessary for folding of its passenger domain. Mol. Microbiol. **47**, 1367–1383.

Oomen, C.J., Van, U.P., Van, G.P., Feijen, M., Tommassen, J., and Gros, P. (2004). Structure of the translocator domain of a bacterial autotransporter. EMBO J. **23**, 1257–1266.

Oresnik,I.J., Ladner, C.L., and Turner, R.J. (2001). Identification of a twin-arginine leader-binding protein. Mol. Microbiol. **40**, 323–331.

Papish, A.L., Ladner, C.L., and Turner, R.J. (2003). The twin-arginine leader-binding protein, DmsD, interacts with the TatB and TatC subunits of the Escherichia coli twin-arginine translocase. J. Biol. Chem. **278**, 32501–32506.

Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P., and Saier, M.H., Jr. (2003). Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. Microbiology **149**, 3051–3072.

Pettersson, J., Nordfelth, R., Dubinina, E., Bergman,T., Gustafsson, M., Magnusson, K.E., and Wolf-Watz, H. (1996). Modulation of virulence factor expression by pathogen target cell contact. Science **273**, 1231–1233.

Pimenta, A.L., Young, J., Holland,I.B., and Blight, M.A. (1999). Antibody analysis of the localisation, expression and stability of HlyD, the MFP component of the E. coli haemolysin translocator. Mol. Gen. Genet. **261**, 122–132.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer,T.F. (1987). Gene structure and extracellular secretion of Neisseria gonorrhoeae IgA protease. Nature **325**, 458–462.

Possot, O.M., Gerard-Vincent, M., and Pugsley, A.P. (1999). Membrane association and multimerization of secreton component pulC. J. Bacteriol. **181**, 4004–4011.

Possot, O.M., Vignon, G., Bomchil, N., Ebel, F., and Pugsley, A.P. (2000). Multiple interactions between pullulanase secreton components involved in stabilization and cytoplasmic membrane association of PulE. J. Bacteriol. **182**, 2142–2152.

Pugsley, A.P. (1993). The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57**, 50–108.

Pugsley, A.P., Francetic, O., Possot, O.M., Sauvonnet, N., and Hardie, K.R. (1997). Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria—a review. Gene **192**, 13–19.

Py, B., Chippaux, M., and Barras, F. (1993). Mutagenesis of cellulase EGZ for studying the general protein secretory pathway in Erwinia chrysanthemi. Mol. Microbiol. **7**, 785–793.

Py, B., Loiseau, L., and Barras, F. (1999). Assembly of the type II secretion machinery of Erwinia chrysanthemi: direct interaction and associated conformational change between OutE, the putative ATP-binding component and the membrane protein OutL. J. Mol. Biol. **289**, 659–670.

Py, B., Loiseau, L., and Barras, F. (2001). An inner membrane platform in the type II secretion machinery of Gram-negative bacteria. EMBO Rep. **2**, 244–248.

Rabel, C., Grahn, A.M., Lurz, R., and Lanka, E. (2003). The VirB4 family of proposed traffic nucleoside triphosphatases: common motifs in plasmid RP4 TrbE are essential for conjugation and phage adsorption. J. Bacteriol. **185**, 1045–1058.

Ray, N., Oates, J., Turner, R.J., and Robinson, C. (2003). DmsD is required for the biogenesis of DMSO reductase in Escherichia coli but not for the interaction of the DmsA signal peptide with the Tat apparatus. FEBS Lett. **534**, 156-160.

Reyrat, J.M., Pelicic, V., Papini, E., Montecucco, C., Rappuoli, R., and Telford, J.L. (1999). Towards deciphering the Helicobacter pylori cytotoxin. Mol. Microbiol. **34**, 197–204.

Ricci, S., Rappuoli, R., and Scarlato, V. (1996). The pertussis toxin liberation genes of Bordetella pertussis are transcriptionally linked to the pertussis toxin operon. Infect. Immun. **64**, 1458–1460.

Robien, M.A., Krumm, B.E., Sandkvist, M., and Hol, W.G. (2003). Crystal structure of the extracellular protein secretion NTPase EpsE of Vibrio cholerae. J. Mol. Biol. **333**, 657–674.

Robinson, C. and Bolhuis, A. (2004). Tat-dependent protein targeting in prokaryotes and chloroplasts. Biochim. Biophys. Acta **1694**, 135–147.

Rohde, J.R., Luan, X.S., Rohde, H., Fox, J.M., and Minnich, S.A. (1999). The Yersinia enterocolitica pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37 degrees C. J. Bacteriol. **181**, 4198–4204.

Rohde, M., Puls, J., Buhrdorf, R., Fischer, W., and Haas, R. (2003). A novel sheathed surface organelle of the Helicobacter pylori cag type IV secretion system. Mol. Microbiol. **49**, 219–234.

Rosch, J. and Caparon, M. (2004). A microdomain for protein secretion in Gram-positive bacteria. Science **304**, 1513–1515.

Rosqvist, R., Magnusson, K.E., and Wolf-Watz, H. (1994). Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. EMBO J. **13**, 964–972.

Rouot, B., varez-Martinez, M.T., Marius, C., Menanteau, P., Guilloteau, L., Boigegrain, R.A., Zumbihl, R., O'Callaghan, D., Domke, N., and Baron, C. (2003). Production of the type IV secretion system differs among Brucella species as revealed with VirB5-and VirB8–specific antisera. Infect. Immun. **71**, 1075–1082.

Sanders, C., Wethkamp, N., and Lill, H. (2001). Transport of cytochrome c derivatives by the bacterial Tat protein translocation system. Mol. Microbiol. **41**, 241–246.

Sandkvist, M. (2001a). Type II secretion and pathogenesis. Infect. Immun. **69**, 3523–3535.

Sandkvist, M. (2001b). Biology of type II secretion. Mol. Microbiol. **40**, 271–283.

Sandkvist, M., Bagdasarian, M., Howard, S.P., and DiRita, V.J. (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in Vibrio cholerae. EMBO J. **14**, 1664–1673.

Sandkvist, M., Hough, L.P., Bagdasarian, M.M., and Bagdasarian, M. (1999). Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in Vibrio cholerae. J. Bacteriol. **181**, 3129–3135.

Sargent, F., Berks, B.C., and Palmer,T. (2002). Assembly of membrane-bound respiratory complexes by the Tat protein-transport system. Arch. Microbiol. **178**, 77–84.

Sargent, F., Bogsch, E.G., Stanley, N.R., Wexler, M., Robinson, C., Berks, B.C., and Palmer,T. (1998). Overlapping functions of components of a bacterial Sec-independent protein export pathway. EMBO J. **17**, 3640–3650.

Sargent, F., Gohlke, U., De, L.E., Stanley, N.R., Palmer,T., Saibil, H.R., and Berks, B.C. (2001). Purified components of the Escherichia coli Tat protein transport system form a double-layered ring structure. Eur. J. Biochem. **268**, 3361–3367.

Saurin, W., Hofnung, M., and Dassa, E. (1999). Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J. Mol. Evol. **48**, 22–41.

Sauvonnet, N. and Pugsley, A.P. (1996). Identification of two regions of Klebsiella oxytoca pullulanase that together are capable of promoting beta-lactamase secretion by the general secretory pathway. Mol. Microbiol. **22**, 1–7.

Sauvonnet, N., Vignon, G., Pugsley, A.P., and Gounon, P. (2000). Pilus formation and protein secretion by the same machinery in Escherichia coli. EMBO J. **19**, 2221–2228.

Savvides, S.N., Yeo, H.J., Beck, M.R., Blaesing, F., Lurz, R., Lanka, E., Buhrdorf, R., Fischer, W., Haas, R., and Waksman, G. (2003). VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion. EMBO J. **22**, 1969–1980.

Schiebel, E., Driessen, A.J., Hartl, F.U., and Wickner, W. (1991). Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64**, 927–939.

Schrammeijer, B., den Dulk-Ras, A., Vergunst, A.C., Jurado, J.E., and Hooykaas, P.J. (2003). Analysis of Vir protein translocation from Agrobacterium tumefaciens using Saccharomyces cerevisiae as a model: evidence for transport of a novel effector protein VirE3. Nucleic Acids Res. **31**, 860–868.

Scott, M.E., Dossani, Z.Y., and Sandkvist, M. (2001). Directed polar secretion of protease from single cells of Vibrio cholerae via the type II secretion pathway. Proc. Natl. Acad. Sci. U. S. A **98**, 13978–13983.

Serruto, D., du-Bobie, J., Scarselli, M., Veggi, D., Pizza, M., Rappuoli, R., and Arico, B. (2003). Neisseria meningitidis App, a new adhesin with autocatalytic serine protease activity. Mol. Microbiol. **48**, 323–334.

Settles, A.M., Yonetani, A., Baron, A., Bush, D.R., Cline, K., and Martienssen, R. (1997). Sec-independent protein translocation by the maize Hcf106 protein. Science **278**, 1467–1470.

Shere, K.D., Sallustio, S., Manessis, A., D'Aversa,T.G., and Goldberg, M.B. (1997). Disruption of IcsP, the major Shigella protease that cleaves IcsA, accelerates actin-based motility. Mol. Microbiol. **25**, 451–462.

Shevchik, V.E., Robert-Baudouy, J., and Condemine, G. (1997). Specific interaction between OutD, an Erwinia chrysanthemi outer membrane protein of the general secretory pathway, and secreted proteins. EMBO J. **16**, 3007–3016.

Shirasu, K., Koukolikova-Nicola, Z., Hohn, B., and Kado, C.I. (1994). An inner-membrane-associated virulence protein essential for T-DNA transfer from Agrobacterium tumefaciens to plants exhibits ATPase activity and similarities to conjugative transfer genes. Mol. Microbiol. **11**, 581–588.

Sijbrandi, R., Urbanus, M.L., ten Hagen-Jongman, C.M., Bernstein, H.D., Oudega, B., Otto, B.R., and Luirink, J. (2003). Signal recognition particle (SRP)-mediated targeting and Sec-dependent translocation of an extracellular Escherichia coli protein. J. Biol. Chem. **278**, 4654–4659.

Soderberg, M.A., Rossier, O., and Cianciotto, N.P. (2004). The type II protein secretion system of Legionella pneumophila promotes growth at low temperatures. J. Bacteriol. **186**, 3712–3720.

Sory, M.P., Boland, A., Lambermont,I., and Cornelis, G.R. (1995). Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. Proc. Natl. Acad. Sci. U. S. A **92**, 11998–12002.

Spudich, G.M., Fernandez, D., Zhou, X.R., and Christie, P.J. (1996). Intermolecular disulfide bonds stabilize VirB7 homodimers and VirB7/VirB9 heterodimers during biogenesis of the Agrobacterium tumefaciens T-complex transport apparatus. Proc. Natl. Acad. Sci. U. S. A **93**, 7512–7517.

Stanley, N.R., Palmer,T., and Berks, B.C. (2000). The twin arginine consensus motif of Tat signal peptides is involved in Sec-independent protein targeting in Escherichia coli. J. Biol. Chem. **275**, 11591–11596.

Stanley, P., Koronakis, V., and Hughes, C. (1991). Mutational analysis supports a role for multiple structural features in the C-terminal secretion signal of Escherichia coli haemolysin. Mol. Microbiol. **5**, 2391–2403.

Stebbins, C.E. and Galan, J.E. (2001). Maintenance of an unfolded polypeptide by a cognate chaperone in bacterial type III secretion. Nature **414**, 77–81.

Straley, S.C., Plano, G.V., Skrzypek, E., Haddix, P.L., and Fields, K.A. (1993). Regulation by Ca2+ in the Yersinia low-Ca2+ response. Mol. Microbiol. **8**, 1005–1010.

Strom, M.S., Bergman, P., and Lory, S. (1993). Identification of active-site cysteines in the conserved domain of PilD, the bifunctional type IV pilin leader peptidase/N-methyltransferase of Pseudomonas aeruginosa. J. Biol. Chem. **268**, 15788–15794.

Sulavik, M.C. **et al.** (2001). Antibiotic susceptibility profiles of Escherichia coli strains lacking multidrug efflux pump genes. Antimicrob. Agents Chemother. **45**, 1126-1136.

Thanabalu,T., Koronakis, E., Hughes, C., and Koronakis, V. (1998). Substrate-induced assembly of a contiguous channel for protein export from E.coli: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. EMBO J. **17**, 6487–6496.

Thayer, M.M., Flaherty, K.M., and McKay, D.B. (1991). Three-dimensional structure of the elastase of Pseudomonas aeruginosa at 1.5–A resolution. J. Biol. Chem. **266**, 2864–2871.

van den, B.B., Clemons, W.M., Jr., Collinson,I., Modis, Y., Hartmann, E., Harrison, S.C., and Rapoport,T.A. (2004). X-ray structure of a protein-conducting channel. Nature **427**, 36-44.

Veenendaal, A.K., van der, D.C., and Driessen, A.J. (2001). Mapping the sites of interaction between SecY and SecE by cysteine scanning mutagenesis. J. Biol. Chem. **276**, 32559–32566.

Veiga, E., Sugawara, E., Nikaido, H., de, L., V, and Fernandez, L.A. (2002). Export of autotransported proteins proceeds through an oligomeric ring shaped by C-terminal domains. EMBO J. **21**, 2122–2131.

Velarde, J.J. and Nataro, J.P. (2004). Hydrophobic residues of the autotransporter EspP linker domain are important for outer membrane translocation of its passenger. J. Biol. Chem. **279**, 31495-31504.

Vergunst, A.C., Schrammeijer, B., den Dulk-Ras, A., de Vlaam, C.M., Regensburg-Tuink,T.J., and Hooykaas, P.J. (2000). VirB/D4-dependent protein translocation from Agrobacterium into plant cells. Science **290**, 970–982.

Vergunst, A.C., van Lier, M.C., den Dulk-Ras, A., and Hooykaas, P.J. (2003). Recognition of the Agrobacterium tumefaciens VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. Plant Physiol **133**, 978–988.

Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F., and Filloux, A. (2001). Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. EMBO J. **20**, 6735–6741.

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M., and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. Science **299**, 262–265.

Voulhoux, R., Taupiac, M.P., Czjzek, M., Beaumelle, B., and Filloux, A. (2000). Influence of deletions within domain II of exotoxin A on its extracellular secretion from Pseudomonas aeruginosa. J. Bacteriol. **182**, 4051–4058.

Vrontou, E. and Economou, A. (2004). Structure and function of SecA, the preprotein translocase nanomotor. Biochim. Biophys. Acta **1694**, 67–80.

Wang, L., Miller, A., Rusch, S.L., and Kendall, D.A. (2004). Demonstration of a specific Escherichia coli SecY-signal peptide interaction. Biochemistry **43**, 13185–13192.

Ward, D.V., Draper, O., Zupan, J.R., and Zambryski, P.C. (2002). Peptide linkage mapping of the Agrobacterium tumefaciens vir-encoded type IV secretion system reveals protein subassemblies. Proc. Natl. Acad. Sci. U. S. A **99**, 11493–11500.

Watarai, M., Andrews, H.L., and Isberg, R.R. (2001). Formation of a fibrous structure on the surface of Legionella pneumophila associated with exposure of DotH and DotO proteins after intracellular growth. Mol. Microbiol. **39**, 313–329.

Wattiau, P., Bernier, B., Deslee, P., Michiels,T., and Cornelis, G.R. (1994). Individual chaperones required for Yop secretion by Yersinia. Proc. Natl. Acad. Sci. U. S. A **91**, 10493–10497.

Wattiau, P., Woestyn, S., and Cornelis, G.R. (1996). Customized secretion chaperones in pathogenic bacteria. Mol. Microbiol. **20**, 255–262.

Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A., and Turner, R.J. (1998). A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell **93**, 93–101.

Yeo, H.J., Savvides, S.N., Herr, A.B., Lanka, E., and Waksman, G. (2000). Crystal structure of the hexameric traffic ATPase of the Helicobacter pylori type IV secretion system. Mol. Cell **6**, 1461–1472.

Yoshida,T., Furuya, N., Ishikura, M., Isobe,T., Haino-Fukushima, K., Ogawa,T., and Komano,T. (1998). Purification and characterization of thin pili of IncI1 plasmids ColIb-P9 and R64: formation of PilV-specific cell aggregates by type IV pili. J. Bacteriol. **180**, 2842–2848.

Zhang, F., Greig, D.I., and Ling, V. (1993). Functional replacement of the hemolysin A transport signal by a different primary sequence. Proc. Natl. Acad. Sci. U. S. A **90**, 4211–4215.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279**, 1718–1721.

Zupan, J., Muth,T.R., Draper, O., and Zambryski, P. (2000). The transfer of DNA from agrobacterium tumefaciens into plants: a feast of fundamental insights. Plant J. **23**, 11–28.

CHAPTER

# 6

# Toxin receptors

*Yasuhiko Horiguchi and Eisuke Mekada*

## GENERAL NOTES ON BACTERIAL TOXIN RECEPTORS

The initial step in the intoxication process by bacterial toxins involves binding to target cells. This is made possible by the presence of specific receptors on the cell surface. Although the molecular nature of the receptors varies depending on the toxins involved, they all bind the toxins, then either transduce particular signals into the cell cytoplasm or are involved in the internalization of the toxins. Cell sensitivity to the toxin is largely determined by the existence of the receptors; therefore these latter provide a molecular basis for the toxins' target cell specificities. As a result, in order to understand toxin actions, molecular analysis of each toxin receptor becomes essential. However, to date, regardless of their substantial roles, only few receptors have been definitively identified (Table 6.1). This is probably because only a small number of receptors are present on the cell surface, and either identification of a particular toxin receptor requires laborious procedures or applicable methods are not known.

For a cellular substance to be accepted as a bacterial toxin receptor, two criteria must be at least satisfied. First, it must be demonstrated that the substance has a property of binding directly and specifically to the toxin. For this purpose, quantitative measurements of the binding must be made. For example, a direct binding assay with the toxin labeled with a radioisotopic or fluorescent probe can be done, and is more preferable than qualitative methods such as combinations of immunoprecipitation and immunoblotting. Indeed, the use of labeled toxins provides much more information about the binding. The Kd or Ka values obtained from the cell-free binding assay might be useful to compare toxin binding affinities to the toxin-susceptible cells. Whether the substance-toxin binding is saturable or unsaturable, this is determined by the direct assay, but it would also be helpful to distinguish the real receptor from the substance that non-specifically binds the toxin. The second criteria addresses cell sensitivity to the toxin. This must depend on the expression level of the substance. For instance, you might have to demonstrate that transfection with the substance cDNA confers toxin sensitivity to the parental toxin-resistant cells, if the putative receptor is a protein.

## HOW DO YOU IDENTIFY BACTERIAL TOXIN RECEPTORS?

Table 6.1 shows a list of bacterial toxin receptors identified to date. Although the process of identification and confirmation of bacterial toxin receptors is far from being simple and contains many episodes for each toxin, we will simply review here critical methods involved in receptor identification. Later on, we particularly provide a detailed description of some toxin receptors.

The cholera toxin receptor was suggested to be GM1 ganglioside in the early 1970s (van Heyningen *et al.*, 1971; Cuatrecasas, 1973; Holmgren, 1973). Attempts to search for the cholera toxin receptor started with findings that the toxins were inactivated by tissue extracts or cell membrane fractions, indicating that these preparations might contain a substance responsible for the

**TABLE 6.1** Bacterial toxin receptors

| Toxin | Receptor | Key method or episode | Key reference |
|---|---|---|---|
| Cholera toxin | GM1 ganglioside | Inhibition of toxic action | van Heyningen *et al.*(1971); Cuatrecasas (1973); Holmgren (1973) |
| Shiga toxin | Gb3 globoside | Inhibition of toxic action | Jacewicz *et al.* (1986) |
| Diphtheria toxin | HB-EGF / 14.5 kDa protein | Expression cloning / Purification | Naglich *et al.* (1992) / Mekada *et al.* (1988) |
| *Pseudomonas* exotoxin A | LDL receptor-related protein | Affinity purification | Thompson *et al.* (1991); Kounnas *et al.* (1992) |
| *E. coli* heat-stable toxin I(a) | Guanylyl cyclase C | PCR with guanylyl cyclase primers | Schulz *et al.* (1990) |
| *Botulinum* toxin type B | Synaptotagmin I and II | Purification | Nishiki *et al.* (1994); Dong *et al.* (2003) |
| Aerolysin | GPI-anchored proteins | Ligand overlay | Cowell *et al.* (1997); Nelson *et al.* (1997) |
| *Clostridium perfringens* enterotoxin | CPE-R / claudin | Expression cloning | Katahira *et al.* (1997a) |
| *Helicobacter pylori* VacA | RPTP α and β | Immunoprecipitation and purification | Yahiro *et al.* (1999); Yahiro *et al.* (2003) |
| *V. cholerae* El Tor hemolysin | Glycophorin B | Preparation of monoclonal antibody to erythrocyte membranes | Zhang *et al.* (1999) |
| Anthrax toxin | TEM8, CMG2 | Expression cloning | Bradley *et al.* (2001) |
| *Bordetella* adenylate cyclase toxin | CD11b / CD18 | Combinations of binding assays | Guermonprez *et al.* (2001) |
| *E. coli* cytotoxic necrotizing factor | Laminin receptor precursor | Yeast two-hybrid system | Chung *et al.* (2003) |

specific binding of the toxins. GM1 ganglioside was then found to inhibit the binding of cholera toxin to the cell membrane and to neutralize toxic action. Subsequently, many studies presented increasing supporting evidence for GM1 ganglioside as the cholera toxin receptor (Hollenberg *et al.*, 1974; Holmgren *et al.*, 1975; Moss *et al.*, 1976). Finally, crystallography of the cholera toxin/GM1 ganglioside complex was available in the 1990s, providing the molecular basis for the toxin-ganglioside interactions (Merritt *et al.*, 1994; Merritt *et al.*, 1998). Gb3 globoside, the Shiga toxin receptor, was also identified in a similar way (Keusch and Jacewicz, 1977; Jacewicz *et al.*, 1986; Acheson and Keusch, 1999). Studies of these toxin receptors indicate that searching for the inhibitory substance to the toxic effect from toxin-sensitive cells is an effective way to identify the toxin receptors. However, this is not sufficient to prove for the real receptor that a cellular substance merely inhibits toxic effects as mentioned above, and in some cases it may meet with a pseudo receptor as exemplified by the nucleotide-like molecules for diphtheria toxin receptor (see page 112 in this chapter).

Protein purification of toxin-binding substances extracted from detergent-solubilized membrane fractions using chromatography, combined with a cell-free binding assay using labeled bacterial toxins, provide a classical but reliable method to identify toxin receptors. Diphtheria toxin receptor and synaptotagmins for botulinum toxin type B were successfully purified from Vero cells and synaptosomal membranes, respectively (Mekada *et al.*, 1988; Mekada *et al.*, 1991; Nishiki *et al.*, 1993, Nishiki *et al.*, 1994). In order to use this method, the following conditions must be fulfilled: (i) binding affinity between toxins and receptors must be fairly high (Kd = $10^{-10} \sim 2 \times 10^{-9}$ M), (ii) rapid and quantitative measurements for toxin-receptor binding using solubilized membrane fractions must be applied, (iii) reasonable amounts of starting material for purification must be available. Recent progress in mass spectrometry (MS) would be quite valuable to identify the protein from partially purified fractions.

The use of expression cloning techniques would provide the most powerful way to identify toxin receptors and their cDNA cloning. In this case, a cDNA library produced by transcripts from toxin-susceptible cells is generally transfected to toxin-resistant (receptor-negative) recipient cells and cells that become toxin-sensitive (or receptor-positive cells by the binding assay with labeled toxin) are picked up. Following screening for receptor-positive cells, the cDNA encoding the receptor is isolated. Screening methods for receptor-positive clones are the key to good results. A replica assay that isolated toxin-sensitive clones was used for heparin-binding EGF-like growth factor (HB-EGF) just like in the case of the diphtheria toxin receptor (Naglich *et al.*, 1992). HB-EGF was later found to be identical to the 14.5 kDa protein of diphtheria toxin receptor that had been purified as mentioned above. Flow cytometry, which seems to be the currently most powerful method for receptor identification, was used

for anthrax toxin receptor (ATR/TEM8) and *Clostridium perfringens* enterotoxin receptor (CPE-R/claudin4) (Katahira *et al*., 1997a; Bradley *et al*., 2001).

Receptor-like protein-tyrosine phosphatases (RPTPs) for *Helicobacter pylori* VacA toxin were identified from sensitive cells by immunoprecipitation and isolated using affinity chromatography (Yahiro *et al*., 1997; Yahiro *et al*., 1999). In this case, the authors found that exposure of VacA to alkaline or acidic conditions increased its binding to the appropriate target cells, which later facilitated the isolation of the receptors.

The receptor for cytotoxic necrotizing factor 1 of *Escherichia coli* was identified using the yeast two-hybrid system. This system is frequently used for DNA cloning (Chung *et al*., 2003), although it may not be generally applicable to membrane proteins like toxin receptors.

*E. coli* heat-stable enterotoxin I (STI) receptor was identified by PCR screening. Since the 1970s, STI has been known to activate guanylate cyclase (Field *et al*., 1978; Hughes *et al*., 1978; Waldman *et al*., 1986). Although most mammalian tissues contain both soluble and particulate forms of guanylate cyclase, the enzyme is predominantly particulate in intestinal mucosa. Thus, particulate guanylate cyclases were thought to be the ST receptor candidates, whilst membrane-type guanylate cyclases known at earlier times were negated as candidates. In 1990, Schulz *et al*. looked for new clones of guanylate cyclases using PCR with degenerate oligonucleotide primers based on conserved sequences in both soluble and plasma membrane forms of the enzyme and template cDNA prepared from small intestinal mucosa, and finally they identified guanylate cyclase C (GC-C) as a STI receptor.

## EXAMPLES OF TOXIN RECEPTORS

### Diphtheria toxin receptor

#### *Diphtheria toxin*

Diphtheria toxin is produced as a single polypeptide chain of 58,348 Da with two disulfide bridges (Collier and Kandel, 1971; Gill and Pappenheimer, 1971). The native toxin is easily hydrolyzed by splitting a peptide bond between one of the disulfide bridges by mild treatment with trypsin-like proteases or furin (Tsuneoka *et al*., 1993) to yield a nicked toxin. On reduction of the disulfide bridges, the nicked toxin is split into two large peptides, fragment A (Mr = 21,167 Da) and fragment B (Mr = 37,199 Da). The toxicity of diphtheria toxin is due to the enzymatic activity of fragment A, catalyzing the transfer of an ADP-ribosyl moiety from NAD to elongation factor 2 (EF-2) (Honjo *et al*., 1968). EF-2 is a GTP-binding protein involved in protein synthesis in eukaryotic cells. ADP-ribosylated EF-2 is unable to mediate polypeptide chain elongation, resulting in inhibition of protein synthesis. Since fragment A alone is unable to penetrate the cell membrane, it cannot reach the cytosol of intact cells without the involvement of fragment B. Fragment B binds to the specific receptor of toxin-susceptible cell membranes (Uchida *et al*., 1972) and facilitates translocation of fragment A into the cytosol.

#### *Cell sensitivity to diphtheria toxin*

Diphtheria toxin is highly toxic to most animals, including rabbits, guinea pigs, and monkeys, as well as humans. The only mammals known to be resistant to the toxin are rats and mice. Cells derived from sensitive species are sensitive to the cytotoxic actions of diphtheria toxin, while cells from toxin-resistant species are quite resistant to its actions (Middlebrook and Dorland, 1977). The resistance of cells to diphtheria toxin is thought to be generally due to the binding and entry process (Yamaizumi *et al*., 1978). Although a number of cellular factors could influence toxin activity, cell sensitivity to diphtheria toxin is primarily determined by the presence of functional diphtheria toxin receptors (Umata *et al*., 2000).

#### *Identification of diphtheria toxin receptor protein*

The existence of a specific receptor for diphtheria toxin on the cell surface was suggested by competition experiments using CRM197, a non-toxic mutant of diphtheria toxin (Itelson and Gill, 1973). Subsequently, binding abilities of $^{125}$I-labeled diphtheria toxin to cultured cells were demonstrated (Middlebrook *et al*., 1978). However, it took about 10 years for the diphtheria toxin receptor to be identified at the protein level from detergent-treated cell fractions (Mekada *et al*., 1988). One difficulty that impeded the identification and purification of the specific receptor for diphtheria toxin from cell fractions was due to the existence of another diphtheria toxin-binding substance present in the membrane fractions (Mekada *et al*., 1988). This non-receptor diphtheria toxin-binding molecule, which contains a ribonucleotide structure, inhibited the binding of the toxin to its specific receptor. Therefore, this non-receptor diphtheria toxin-binding substance was initially mistaken for the real receptor.

A solution to the problem was obtained from binding assays with CRM197. CRM197 differs from the wild-type toxin in one amino acid residue of fragment A (Giannini *et al*., 1984). CRM197 does not bind nucleotides, the non-receptor diphtheria toxin-binding substances (Mekada *et al*., 1988) but binds to diphtheria

toxin receptor with an affinity similar to or greater than that of diphtheria toxin (Mekada and Uchida, 1985). A binding assay with $^{125}$I-labeled CRM197 enabled to demonstrate the specific binding to the diphtheria toxin receptor in a membrane preparation from Vero cells (Mekada *et al*., 1988) with a Kd value of 2.4 nM, which was quite similar to that obtained with intact Vero cells. Using a combination of several chromatographic steps, the diphtheria toxin receptor was purified in the presence of detergents (Mekada *et al*., 1991). The purified receptor essentially showed a single band of 14.5 kDa by SDS-PAGE. Evidence that the 14.5 kDa protein was the receptor, or at least a part of the receptor, was obtained by immunoprecipitation with diphtheria toxin and anti-diphtheria toxin antibody from partially purified receptor fractions or by blotting using $^{125}$I-CRM197 as a probe (Mekada *et al*., 1988). Although the 14.5 kDa protein was the major form isolated from Vero cell membrane lysates, other sizes of diphtheria toxin-binding molecules (ac 20 and 17 kDa) were detected in different fractions using CM-Sepharose ion-exchange chromatography (Mekada *et al*., 1991). The 14.5 kDa protein and the 17 and 20 kDa proteins were suggested to derive from a single precursor protein, as discussed below.

### *Cloning of the diphtheria toxin receptor gene*

cDNA encoding the diphtheria toxin receptor was isolated as a diphtheria toxin sensitivity gene by expression cloning (Naglich *et al*., 1992). Mouse L cells were transfected with a cDNA library obtained from monkey kidney (Vero) cells. Transfectants that were sensitive to diphtheria toxin were isolated using a replica plate assay (Naglich and Eidels, 1990). cDNA (pDTS) was recovered from the diphtheria toxin–sensitive transfectants. Mouse L cells transfected with this cDNA became sensitive to diphtheria toxin and displayed diphtheria toxin–binding molecules on the cell surface that had the characteristics of the diphtheria toxin receptor (Naglich *et al*., 1992). The predicted protein product of pDTS was 185 amino acids, showed a quite basic isoelectric point, and had characteristics of an integral membrane protein. These characteristics were consistent with the characteristics of the diphtheria toxin receptor protein isolated by chromatography (Mekada *et al*., 1991).

pDTS is identical to a known heparin-binding growth factor, HB-EGF (Naglich *et al*., 1992), initially identified by Higashiyama (Higashiyama *et al*., 1991). Further evidence that the product of pDTS was the diphtheria toxin receptor has been obtained from direct binding experiments with diphtheria toxin and a recombinant human HB-EGF (Iwamoto *et al*., 1994). $^{125}$I-diphtheria toxin bound to immobilized recombinant HB-EGF with an affinity similar to that of intact Vero cells (Kd of about 1 nM). These results clearly show that the diphtheria toxin receptor is HB-EGF.

### *Structure and function of the diphtheria toxin receptor*

Diphtheria toxin receptor/HB-EGF cDNA encodes a protein of 208 amino acids (Higashiyama *et al*., 1991; Naglich *et al*., 1992). This protein consists of a characteristic signal sequence of 23 amino acid residues, a presumed extracellular domain of 136 residues (24–159), a putative transmembrane domain of 25 residues (160–184), and a carboxyl terminal cytoplasmic domain of 24 residues (185–208). The mature protein, after cleavage of the signal peptide, seems to be comprised of 185 amino acids with a calculated molecular weight of 20,652 Da. The extracellular domain includes two characteristic features: (i) an EGF-like domain with six cysteine residues with highly conserved spacing and (ii) a heparin-binding domain with a highly basic stretch of amino acid residues upstream from the EGF-like domain.

Because HB-EGF is expressed in multiple tissues, i.e., in rats, mice, and humans, with a very similar tissue distribution (Abraham *et al*., 1993), the question arises as to why cells from rats and mice are resistant to diphtheria toxin. Transfection of human diphtheria toxin receptor/HB-EGF cDNA into mouse L cells confers sensitivity to diphtheria toxin, but transfection of mouse HB-EGF cDNA does not (Mitamura *et al*., 1995). These results indicate that mouse HB-EGF does not serve as a functional receptor for diphtheria toxin because of amino acid substitution. Transfection studies of a series of human/mouse HB-EGF chimeras indicated that the EGF-like domain of the human HB-EGF is essential for diphtheria toxin binding and diphtheria toxin sensitivity (Mitamura *et al*., 1995).

Results from human/mouse HB-EGF chimeras provide information on specific important amino acids for diphtheria toxin-diphtheria toxin receptor interactions. There are 10 amino acid differences between the EGF-like domain of human diphtheria toxin receptor/HB-EGF and that of mouse HB-EGF. Transfection experiments of human/mouse HB-EGF chimeras into mouse L cells revealed that the biggest effects were observed with E141H, and the second biggest effects were with F115Y and L127F (Mitamura *et al*., 1997). A computer model of the tertiary structure of the EGF-like domain of human diphtheria toxin receptor/HB-EGF, based on the tertiary structure of TGFα, predicted that three amino acid residues critical for diphtheria toxin-binding activity, Phe115, Leu127, and Glu141, are all located on the same face of the EGF-like domain, suggesting that this face of the diphtheria toxin receptor/proHB-EGF interacts with the receptor-binding

domain of diphtheria toxin. The crystal structure of diphtheria toxin-HB-EGF (Louie *et al*., 1997), which is quite consistent with the above model, provided a more defined description of its complex structure.

### *Molecules associated with the diphtheria toxin receptor and modulating receptor function*

*DRAP27/CD9*

DRAP27 (*D*iphtheria toxin *R*eceptor-*A*ssociated *P*rotein 27) was originally identified as the antigen of a monoclonal antibody that inhibits the binding of diphtheria toxin to intact Vero cells. Cloning of DRAP27 cDNA revealed that DRAP27 has 228 amino acids containing four putative transmembrane domains and that it is identical to the CD9 antigen (Mitamura *et al*., 1992). The role of DRAP27/CD9 in diphtheria toxin binding and diphtheria toxin sensitivity has been studied by transfecting DRAP27 cDNA into cells. DRAP27/CD9 alone had no particular function for enhancing the sensitivity to diphtheria toxin. However, when L cells were transfected with both DRAP27/CD9 and diphtheria toxin receptor cDNA, transfectants expressing both diphtheria toxin receptor and DRAP27 had a 15 times greater cell surface diphtheria toxin receptor number and were 20 times more sensitive to diphtheria toxin than stable L-cell transfectants expressing diphtheria toxin receptor alone, though the cell lines contained similar levels of diphtheria toxin receptor mRNA. Thus, it is clear that DRAP27/CD9 upregulates diphtheria toxin receptor number and diphtheria toxin sensitivity through protein-protein interaction, although the upregulation mechanism is still elusive.

*Heparin-like molecules*

Diphtheria toxin receptor/proHB-EGF shows high affinity to heparin. It associates with heparan sulfate proteoglycan at the heparin-binding domain (HBD). Diphtheria toxin receptor binds diphtheria toxin at the EGF-like domain, and this domain is sufficient to bind diphtheria toxin, while binding of heparin-like molecules to HBD is necessary for full binding activity of diphtheria toxin receptor to diphtheria toxin when assayed in intact cells (Shishido *et al*., 1995). ProHB-EGF mutants lacking HBD have higher affinity for diphtheria toxin than that of wild type proHB-EGF in the absence of heparin-like molecules. By adding heparin or heparan sulfate, diphtheria toxin binding was greatly increased in wild type proHB-EGF, but didn't increase a lot in proHB-EGF mutants lacking HBD (Takazaki *et al*., 2004). These results indicate that HBD is not necessary for binding to diphtheria toxin, but rather plays a role as a negative regulator for the binding of diphtheria toxin to the EGF-like domain of proHB-EGF. Heparin or heparan sulfate proteoglycans would cancel or neutralize the negative effect of HBD through binding to the HBD domain.

### *Physiological role of the diphtheria toxin receptor*

Diphtheria toxin receptor, i.e., HB-EGF, is cleaved upstream of the transmembrane domain by a cell-associated protease, and the extracellular domain is released into the medium as a soluble growth factor (Goishi *et al*., 1995). The secreted HB-EGF protein is also processed at multiple sites at the N-terminal portion (Nakagawa *et al*., 1996). Thus, multiple forms of HB-EGF with different molecular sizes are secreted. HB-EGF belongs to the EGF family of growth factors (Carpenter and Cohen, 1979). It binds to and gives a mitogenic signal through the EGF receptor and ErbB-4 (Elenius *et al*., 1997). The soluble form of HB-EGF is a potent mitogen and chemoattractant for a number of cell types, including vascular smooth muscle cells, fibroblasts, and keratinocytes (Higashiyama *et al*., 1993; Raab and Klagsbrun, 1997). HB-EGF has been implicated in a number of physiological and pathological processes, which include wound healing (Tokumaru *et al*., 2000), cardiac hypertrophy (Asakura *et al*., 2002), smooth muscle cell hyperplasia (Miyagawa *et al*., 1995), kidney collecting duct morphogenesis (Takemura *et al*., 2001), blastocyst implantation (Das *et al*., 1994), pulmonary hypertension (Lemjabbar and Basbaum, 2002), and oncogenic transformation (Fu *et al*., 1999). Recent studies on HB-EGF null mice ($HB^{del/del}$) revealed that HB-EGF is essential for normal heart function and $HB^{del/del}$ developed severe heart failure with grossly enlarged ventricular chambers and enlarged cardiac valves (Iwamoto *et al*., 2003).

Processing of the juxtamembrane domain of HB-EGF, so-called ectodomain shedding, is quite important for the function of HB-EGF (Yamazaki *et al*., 2003), and it is regulated by various extracellular stimuli and intracellular signaling (Izumi *et al*., 1998; Umata *et al*., 2001; Takenobu *et al*., 2003). Recent studies indicate that HB-EGF, especially in its soluble form, plays pivotal roles in tumor growth of cancer cells (Miyamoto *et al*., 2004). Thus, HB-EGF would be a potential target for cancer therapy.

## *Clostridium perfringens* enterotoxin (CPE) receptor

### Clostridium perfringens *enterotoxin*

CPE is a member of the pore-forming toxins, which destroy mammalian target cells by perturbing selective permeability of the plasma membrane. CPE is a single chain polypeptide that consists of 319 amino acids,

which is functionally separable into an N-terminal domain involved in pore formation and a C-terminal domain responsible for binding to target cells (Horiguchi *et al*., 1987; Hanna *et al*., 1991; Kokai-Kun *et al*., 1999). It is considered that after binding to target cells, large protein complexes comprised of CPE and cellular substances form in the cell membrane, leading to cell destruction.

### *CPE receptor*

The toxin does not affect erythrocytes, but a limited number of nucleated cell types to which CPE specifically binds (McDonel and McClane, 1979; Horiguchi *et al*., 1985), implying that the toxin utilizes a specific receptor, which is not ubiquitous in mammalian cells. Early studies demonstrated that pretreatment of rabbit intestinal cells (or their membranes) with proteases but not with neuraminidase, reduced or abolished CPE binding, indicating that the receptor has a protein moiety (McDonel, 1980; Wnek and McClane, 1986). Katahira *et al*. (1997), using expression cloning techniques, first reported the identification of the receptor (Katahira *et al*., 1997a). A cDNA encoding a 22 kDa protein was cloned from a cDNA library of CPE-sensitive Vero cells by screening transfected cells that bound to the C-terminal binding domain of the toxin. The nucleotide sequence of this gene revealed that the 22 kDa protein (designated CPE-R) consisting of 209 amino acids is highly hydrophobic, has four putative transmembrane domains, and shows homologies with the rat androgen withdrawal apoptosis protein RVP1 and the mouse oligodendrocyte specific protein (OSP). The CPE-R gene was found to be expressed only in the CPE-sensitive Vero, Hep3B, and Intestine 407 cells, but not in the CPE-resistant L929, K562, and JY cells. Moreover, transfection with the CPE-R gene conferred sensitivity to CPE-resistant L929 cells. These results clearly indicated that CPE-R was the functional receptor for CPE, although its physiological role remained unknown at that time. RVP1 was also evidenced to serve as another receptor for CPE (Katahira *et al*., 1997b).

### *Physiological function of CPE receptor*

Shortly after Katahira *et al*.'s report, it was found that CPE-R and its related genes constitute a novel multigene family, of which members are integral components of tight-junction strands at intercellular borders. Furuse *et al*. (1998) first identified two distinct components of tight junctions, which show similarities to CPE-R and RVP1, and designated them claudin-1 and claudin-2 (Furuse *et al*., 1998). Subsequently, database searches revealed the knowledge of six claudin-like proteins, including CPE-R and RVP1, and now, 24 members of the claudin family are known (Morita *et al*., 1999a; Morita *et al*., 1999b; Tsukita *et al*., 2001). Of these members, RVP1 and CPE-R were designated as claudin-3 and claudin-4, respectively. Furthermore, claudin-6, -7, -8, 14 were found to function as CPE-receptors with varied binding affinities (Table 6.2) (Fujita *et al*., 2000).

### *Structure and function*

Claudins commonly have four transmembrane domains and two extracellular loops with intracellular N- and C-termini. CPE recognizes the second extracellular loop of claudin-3 and probably other sensitive claudins (Fujita *et al*., 2000). However, neither the consensus sequence nor steric structure recognized by CPE has been defined in the second extracellular loop, which comprises fewer than 20 amino acids. On the other hand, the binding domain of CPE is limited to the C-terminal 30 amino acids (Hanna *et al*., 1991). In fact, the C-terminal fragment of CPE (C-CPE) bound to claudins on cells without any destructive effects with a Ka value equivalent to that of the full-length CPE (Horiguchi *et al*., 1987; Katahira *et al*., 1997a). Furthermore, it was found that C-CPE reversibly reduced the tight junction barrier of the epithelial layer made by MDCK I cells and degraded the network of tight junction strands, removing claudin-4/CPE-R from tight junctions (Sonoda *et al*., 1999). As a result, these observations provided conclusive evidence that claudins are, both structurally and functionally, involved in the construction of tight junctions.

### *Receptor-associating molecule*

Besides claudins, early attempts in the 1980s using affinity chromatography and immunoprecipitation methods demonstrated that 50–60 kDa and 70 kDa proteins could be isolated from CPE sensitive but not resistant cells (Wnek and McClane, 1983; Sugii and Horiguchi, 1988). These proteins were considered to be associated with the complex that forms after CPE binds to target cells (Wieckowski *et al*., 1994). The 70 kDa protein was identified as occludin, which is also an integral membrane protein localized at tight junctions (Singh *et al*., 2000). Occludin, however, endowed the CPE-resistant Rat-1/R12 fibroblasts with neither the sensitivity nor the ability to bind CPE.

## Botulinum neurotoxin receptor

### *Botulinum neurotoxin*

*Clostridium botulinum* neurotoxins (BoNTs) are known to cause muscle paralysis by blocking neurotransmitter release from nerve terminals at neuromuscular junctions. They can be classified into seven types from A to

**TABLE 6.2** Characteristic features and binding ability to CPE of claudins

| Claudin[a] | Distinctive characteristics[b] | Affinity to CPE (Ka)[c] |
|---|---|---|
| 1 | Present in high-resistance epithelia. | -[d] |
| 2 | Present in leaky epithelia. | - |
| 3 | Also known as RVP1. Present in the tighter segments of nephrons. | $4.6 \times 10^7\ M^{-1}$ |
| 4 | Present in the tighter segments of nephrons. | $7.9 \times 10^7\ M^{-1}$ |
| 5 | Alternative name for TMVCF, as it is frequently deleted in Velo cardio facial syndrome. Constitutes TJ strands in endothelial cells. | - |
| 6 | Present in embryonic epithelia. | $9.7 \times 10^7\ M^{-1}$ |
| 7 | Down-regulated in head and neck squamous cell carcinomas. | $8.8 \times 10^7\ M^{-1}$ |
| 8 | Present in the tighter segments of nephrons. | $1.0 \times 10^6\ M^{-1}$ |
| 10 | Not well characterized. | - |
| 11 | Also named OSP. Present in oligodendrocytes and Sertoli cells. | ND[e] |
| 14 | Expressed in the sensory epithelium of the organ of Corti. | $3.6 \times 10^6\ M^{-1}$ |
| 15 | Present in endothelial cells. | ND |
| 16 | Also known as paracelin-1. Critical for $Mg^{2+}$ and $Ca^{2+}$ resorption in the human thick ascending limb of Henle. | ND |
| 18 | Expressed in lung and stomach. | ND |

[a]Claudins 9, 12, 13, 17 and 19–24 still remain to be characterized and it is still unknown if they are CPE receptors.
[b]Refer to González-Mariscal *et al.* (2003).
[c]Refer to Katahira *et al.* (1997) and Fujita *et al.*. (2000).
[d]-; Not bound to CPE
[e]ND; Not determined

G on the basis of antigenic differences. All types of toxins are single chain polypeptides of ~150 kDa, which consist of an N-terminal light chain (approximately 50 kDa) and a C-terminal heavy chain (approximately 100 kDa). The heavy chain can be further segregated into an N-terminal ($H_N$) and a C-terminal ($H_C$). BoNTs bind to presynaptic membranes via the $H_C$ domain, and the light chain is intracellularly translocated and inhibits neurotransmitter release through its endopeptidase activities, which cleave the members of the synapse-specific proteins called SNAREs.

### *Identification of the receptor*

BoNTs specifically bind to cholinergic nerve terminals. Polysialogangliosides such as GD1a and GT1b, which are ubiquitously present in nerve tissues, were analyzed as receptor candidates, because they bound and inactivated BoNTs (Simpson and Rapport, 1971; Kitamura *et al*., 1979). However, the direct interaction between the toxin and the gangliosides hardly occurred under physiological conditions (Kozaki *et al*., 1984). Binding studies revealed that the toxin-binding molecules on the pre-synaptic membranes are different among toxin types (Kozaki, 1979; Black and Dolly, 1986), whereas ganglioside GT1b equally inactivates many types of BoNTs (Kozaki *et al*., 1984). Thus, whether the gangliosides are true receptors had remained uncertain.

Synaptotagmin, a synaptic vesicle membrane protein, was first isolated as a potential receptor from rat brain synaptosomes (Nishiki *et al*., 1993; Nishiki *et al*., 1994). BoNT type B bound to synaptotagmin isoforms I and II in the presence of gangliosides GT1b and GD1a with dissociation constants of 2.3 and 0.23 nM, respectively (Nishiki *et al*., 1996a). Synaptotagmin II binding to BoNT type B with high affinity is known to be heavily expressed in peripheral nerve cells, which is consistent with the fact that BoNTs act at neuromuscular junctions. Synaptotagmin, which resides in the synaptic vesicles, possesses a single transmembrane domain with short intravesicular N- and cytoplasmic C-termini. BoNT type B is believed to bind to the N-terminus of synaptotagmin through the $H_C$ domain that is also known to mediate the binding to gangliosides (Kamata *et al*., 1986; Kozaki *et al*., 1989). Ganglioside GT1b was found to be associated with the transmembrane domain of synaptotagmin probably via its ceramide moiety (Kozaki *et al*., 1998). Therefore, it is likely that the N-terminus of synaptotagmin and GT1b, which are associated within the cell membrane, cooperatively compose a recognition site for BoNT type B binding.

In 2003, Dong *et al*. proved that synaptotagmin is the functional receptor. In their study, Dong *et al*. used PC12 cells, which intrinsically express no synaptotagmin II and contain only low levels of gangliosides. They demonstrated that BoNT type B entered PC12 cells that had previously been loaded with gangliosides and cleaved intracellular synaptobrevin, a substrate for the toxin, but it did not intoxicate another

PC12 cell line that was lacking synaptotagmin I and II. Transfection of PC12 cells with the synaptotagmin II gene rendered the cells sensitive to the toxin even without ganglioside preloading.

Earlier studies demonstrated that intoxication of motoneurons by BoNTs emerged only after a certain duration of stimulation-induced muscle constrictions. Synaptotagmin provided a conceivable interpretation of this phenomenon. The intravesicular N-terminus of synaptotagmin is exposed to the extracellular milieu during exocytosis and recycling of synaptic vesicles (Matteoli *et al*., 1992; Nishiki *et al*., 1996b; Kozaki *et al*., 1998; Dong *et al*., 2003). In other words, synaptotagmins should appear *de novo* outside cells only when exocytosis (neurotransmitter release) occurs and synaptic vesicle membranes fuse with the plasma membrane; and then, the toxin bound to synaptotagmin should be internalized in concert with membrane recycling. In fact, it was shown that BoNT type B entered cells together with synaptotagmin in response to stimulation (Dong *et al*., 2003). Recently, it was reported that synaptotagmins I and II also serve as receptors for BoNT type G. Receptors for other types of BoNT are still unknown.

## *Helicobacter pylori* VacA toxin receptor

### *VacA toxin*

*Helicobacter pylori* vacuolating cytotoxin (VacA), which is known to cause characteristic vacuolation in toxin-sensitive cells, is synthesized as a 140 kDa precursor and is processed into the mature 95 kDa toxin during secretion. The mature toxin is further cleaved into an N-terminal 37 kDa fragment (p37) and a C-terminal 58 kDa fragment (p58), which both remain non-covalently associated. p58 is considered to conduct the binding and internalization steps in the toxic actions. The VacA gene shows variations in two distinct molecular regions; one resides in the signal sequence region (genotypes s1 and s2), and the other resides in the mid region of p58 (genotypes m1 and m2). Based on combinations of these genotypes, VacA can be classified into four types: s1/m1, s1/m2, s2/m1, and s2/m2, of which s1/m1 is the most toxic. In addition, it has been reported that m1 and m2 types of VacA show different specificities of target cells (Pagliaccia *et al*., 1998). Attempts to identify the VacA receptor have been conducted using m1-VacA.

### *VacA toxin receptor*

Two distinct proteins with 250 kDa and 140 kDa were first identified by immunoprecipitation as specific proteins associated with active VacA and subsequently isolated chiefly by lectin affinity chromatography. Partial amino acid sequences of the isolated proteins were consistent with those of receptor-like protein tyrosine phosphatase (RPTP) β and α, respectively (Yahiro *et al*., 1997; Yahiro *et al*., 1999; Yahiro *et al*., 2003). HL-60 cells that are originally resistant to VacA became sensitive during differentiation into macrophage or monocyte-like cells by stimulation with phorbol ester. This was accompanied with the expression of RPTPβ (de Bernard *et al*., 1998; Padilla *et al*., 2000). Furthermore, VacA resistant BHK-21 cells gained the ability to respond to the toxin after being transfected with RPTPβ cDNA (Padilla *et al*., 2000). RPTPα was isolated from G401 Wilms' human kidney tumor cells, which do not express RPTPβ. Inhibition of RPTPα expression by an antisense oligonucleotide reduced the levels of internalization and intoxication with VacA in G401 cells. Thus, it was proved that RPTPα and β are the functional receptors for VacA (Yahiro *et al*., 2003).

### *Structure and function*

There are four splicing variants of RPTPβ: the full- length transmembrane form RPTPβ-A, the short transmembrane form RPTPβ-B, and two soluble short forms called *phosphacans*. Among them, both the transmembrane proteins, RPTPβ-A and RPTPβ-B, serve as VacA receptors. RPTPs including RPTPα possess sugar moieties in their molecules. Because treatments of target cells with neuraminidase and of RPTPβ with neuraminidase or *O*-glycosidase diminished VacA binding, it becomes clear that the sugar moiety must be functioning as part of the receptors. Recently, the main region of RPTPβ-B that interacts with VacA was defined to five residues (QTTQP) at position 747–751, containing two threonines and potential *O*-glycosylation sites (Yahiro *et al*., 2004). This same region is commonly retained in RPTPβ-A. VacA caused gastric tissue damage leading to gastric ulcers in wild type mice but not in RPTPβ-deficient mice. At the cellular level, VacA caused detachment from the basement membrane in gastric epithelial cells in wild type mice but not in RPTPβ-deficient mice, although it caused the vacuolation in both types of cells. Pleiotrophin, an endogenous ligand for RPTPβ, also caused similar gastritis only in wild type mice. These results imply that: (i) VacA induces gastric damage through RPTPβ-dependent signaling pathways, (ii) pleiotrophin also acts as a stimulator, (iii) RPTPβ is not essential for the VacA-induced vacuolation in cells (Fujikawa *et al*., 2003). The binding region of RPTPβ for pleiotrophin is unlikely to be the same as that for VacA (Yahiro *et al*., 2004). Besides the vacuolating activity and induction of gastric cell damage, VacA is known to exert various biological activities, such as proinflammatory or immunosuppressive activities on T cells and apoptosis-inducing activity (Boncristiano *et al*., 2003; Gebert *et al*.,

2003; Sundrud *et al*., 2004). It remains to be determined whether RPTPs are involved in these VacA activities, although Boncristinano *et al*. (2003) implied that RPTPs are partly involved in the inhibition of T cell proliferation.

## Anthrax toxin receptor

### *Anthrax toxin*

Anthrax toxin is composed of three subunits: edema factor (EF), lethal factor (LF), and protective antigen (PA) (Leppla, 1995). EF is an adenylate cyclase, while LF is a metalloprotease specific for MAPK kinases. Although EF and LF are responsible for the toxicity of the anthrax toxin, these subunits cannot exert their effects in the absence of PA. PA is a receptor-binding protein that forms a pore in the endosomal membrane, enabling EF and LF to cross into the cytosol. The current model for cellular intoxication of anthrax toxin involves a multi-step mechanism. In the first step, the full-length 83 kDa form of PA (PA83) binds a host cell surface receptor. PA83 is cleaved by a furin-like protease on the cell surface, yielding the 63 kDa form of PA (PA63) (Molloy *et al*., 1992). PA63 then oligomerizes into a heptameric, receptor-bound prepore (Petosa *et al*., 1997). Oligomerized PA also binds LF and/or EF. The toxin/receptor complex is internalized by receptor-mediated endocytosis and delivered into an acidic endosomal compartment (Abrami *et al*., 2003). The low pH present inside the endosome induces a conformational change of the prepore, which leads to its insertion in the endosomal membrane and translocation of EF and LF into the cytoplasm (Blaustein *et al*., 1989).

### *Identification of anthrax toxin receptor*

Specific binding of PA to anthrax toxin was studied by using $^{125}$I-labeled PA (Singh *et al*., 1991). $^{125}$I-PA binds to specific cell surface receptors of CHO cells and mouse macrophages in a saturated manner with a single class of binding having a Kd of about 1 nM (Escuyer and Collier, 1991; Friedlander *et al*., 1993). Macrophages from different mouse strains vary in their sensitivity to anthrax toxin (PA plus LF), but show similar specific binding to PA, indicating that macrophage cell sensitivity to this toxin does not depend on the presence of functional anthrax toxin receptors, but is possibly due to the ubiquitous expression of the receptors (Friedlander *et al*., 1993).

Anthrax toxin receptor was identified by expression cloning using toxin-resistant mutant cells (Bradley *et al*., 2001). CHO-K1 cells are sensitive to anthrax toxin. To identify the anthrax toxin receptor, Bradley *et al*. (2001) first isolated receptor-deficient mutant cells by selecting for PA and LFN-DTA (a fusion protein composed of the N-terminal 255 amino acids of LF linked to the catalytic subunit of diphtheria toxin). The isolated mutant cells were defective in PA binding, suggesting that these cells lost expression of a putative PA receptor gene. Genetic complement analysis using a retrovirus-based cDNA library from human HeLa cells with a combination of selection of cells with a significant signal of PA binding detected by flow cytometry, identified a cDNA clone encoding the anthrax toxin receptor (ATR). Transfection of the ATR cDNA restored not only PA binding to CHO-R1.1 cells, but also sensitivity to LFN-DTA toxin in the presence of PA. Furthermore, ATR cDNA enhanced the binding of PA to parental CHO-K1 cells.

The cloned ATR is encoded by the tumor endothelial marker 8 (TEM8) gene, which is expressed in a wide variety of tissues. Although the physiological function of ATR/TEM8 is not yet known, this gene is suggested to have some roles in neovascularization. At least three different ATR/TEM8 protein isoforms have been described that are produced by alternative splicing of the TEM8 gene transcript, and two of the three isoforms (splice variants 1-2, or sv1-2) are suggested to function as ATRs (Scobie *et al*., 2003).

In addition to ATR/TEM8, capillary morphogenesis protein 2 (CMG2), originally known as a gene expressed in human placental tissues (Scobie *et al*., 2003), was also identified as a second PA receptor. Several different isoforms of CMG2 protein, which are generated by alternative splicing from CMG2 gene, are known; and at least CMG2$^{489}$, one isoform of the spliced variants, confers PA binding activity and sensitivity to LFN-DTA toxin in the presence of PA on CHO-R1.1 cells to a degree similar to that observed in ATR/TEM8. CMG2 gene, as well as ATR/TEM8 gene, is expressed in a variety of human tissues, suggesting that CMG2 protein is also involved in pathogenesis by anthrax toxin.

### *Structure and function*

The extracellular domains of ATR/TEM8 and CMG2 commonly contain a region that is highly related to von Willebrand factor type A domains (VWA domains), also called *integrin inserted domains* (I domains). The VWA/I domain of CMG2 is 60% identical to the VWA/I domain of ATR/TEM8. These domains are known to be important for protein-protein interactions and constitute ligand-binding sites for integrins. VWA/I domains frequently contain a metal ion–dependent adhesion site (MIDAS) motif. PA directly binds to the VWA/I domains of ATR/TEM8 and CMG2 in a metal ion–dependent manner (Bradley *et al*., 2001; Scobie *et al*., 2003).

Recently, the crystal structure of the single VWA domain of CMG2 (Lacy *et al*., 2004b) and the CMG2-PA complex structure (Lacy *et al*., 2004a; Santelli *et al*., 2004) were reported. The structure indicates that the binding surface of PA with CMG2 quite resembles that of collagen-integrin interaction, and also revealed the nature of interaction of PA with CMG2 at an atomic level, providing information for creating a new anthrax antitoxin.

## CONCLUSION

Although characterization of many bacterial toxin receptors is still not clarified, it is now clear that all bacterial toxin receptors identified to date play pivotal roles in host cell function, as well as intoxication by bacterial toxins. Membrane proteins that are ubiquitously present and essential for host cell membrane would be favorable for bacteria to exert their toxic actions to a large extent, and thus such membrane proteins must be particularly selected as toxin receptors. Identification and molecular characterization of their receptors are not only essential to understanding the intoxication mechanism of bacterial toxins, but also valuable for analyzing native functions of host cell proteins. Recent progress on analytical methods for dealing with small protein amounts seems to have eased the identification of toxin receptors. It is hoped that future efforts will identify receptors of most bacterial toxins.

## REFERENCES


Abraham, J.A., Damm, D., Bajardi, A., Miller, J., Klagsbrun, M. and Ezekowitz, R.A. (1993). Heparin-binding EGF-like growth factor: characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. *Biochem. Biophys. Res. Commun*. **190**, 125–133.

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003). Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated, clathrin-dependent process. *J. Cell. Biol*. **160**, 321–328.

Acheson, D.W.K. and Keusch, G.T. (1999). The family of Shiga toxins. In: The *Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer). pp. 229–242. Academic Press, San Diego.

Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M. and Higashiyama, S. (2002). Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med*. **8**, 35–40.

Black, J.D. and Dolly, J.O. (1986). Interaction of 125I-labeled botulinum neurotoxins with nerve terminals. (I) Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. *J. Cell Biol*. **103**, 521–534.

Blaustein, R.O., Koehler, T.M., Collier, R.J. and Finkelstein, A. (1989). Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc. Natl. Acad. Sci. USA* **86**, 2209–2213.

Boncristiano, M., Paccani, S.R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., Amedei, A., D'Elios, M.M., Telford, J.L. and Baldari, C.T. (2003). The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J. Exp. Med*. **198**, 1887–1897.

Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, J.A. (2001). Identification of the cellular receptor for anthrax toxin. *Nature* **414**, 225–229.

Carpenter, G. and Cohen, S. (1979). Epidermal growth factor. *Annu. Rev. Biochem*. **48**, 193–216.

Chung, J.W., Hong, S.J., Kim, K.J., Goti, D., Stins, M.F., Shin, S., Dawson, V.L., Dawson, T.M. and Kim, K.S. (2003). 37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake. *J. Biol. Chem*. **278**, 16857–16862.

Collier, R.J. and Kandel, J. (1971). Structure and activity of diphtheria toxin. I. Thiol-dependent dissociation of a fraction of toxin into enzymically active and inactive fragments. *J. Biol. Chem*. **246**, 1496–1503.

Cowell, S., Aschauer, W., Gruber, H.J., Nelson, K.L. and Buckley, J.T. (1997). The erythrocyte receptor for the channel-forming toxin aerolysin is a novel glycosylphosphatidylinositol-anchored protein. *Mol. Microbiol*. **25**, 343–350.

Cuatrecasas, P. (1973). Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **12**, 3558–3566.

Das, S.K., Wang, X.N., Paria, B.C., Damm, D., Abraham, J.A., Klagsbrun, M., Andrews, G.K. and Dey, S.K. (1994). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development* **120**, 1071–1083.

de Bernard, M., Moschioni, M., Papini, E., Telford, J.L., Rappuoli, R. and Montecucco, C. (1998). TPA and butyrate increase cell sensitivity to the vacuolating toxin of *Helicobacter pylori*. *FEBS Lett*. **436**, 218–222.

Dong, M., Richards, D.A., Goodnough, M.C., Tepp, W.H., Johnson, E.A. and

Chapman, E.R. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell. Biol*. **162,** 1293–1303.

Elenius, K., Paul, S., Allison, G., Sun, J. and Klagsbrun, M. (1997). Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J*. **16,** 1268–1278.

Escuyer, V. and Collier, R.J. (1991). Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. *Infect. Immun*. **59,** 3381–3386.

Field, M., Graf, L.H., Jr., Laird, W.J. and Smith, P.L. (1978). Heat-stable enterotoxin of Escherichia coli: *in vitro* effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proc. Natl. Acad. Sci. USA* **75,** 2800–2804.

Friedlander, A.M., Bhatnagar, R., Leppla, S.H., Johnson, L. and Singh, Y. (1993). Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect. Immun*. **61,** 245–252.

Fu, S., Bottoli, I., Goller, M. and Vogt, P.K. (1999). Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation. *Proc. Natl. Acad. Sci. USA* **96,** 5716–5721.

Fujikawa, A., Shirasaka, D., Yamamoto, S., Ota, H., Yahiro, K., Fukada, M., Shintani, T., Wada, A., Aoyama, N., Hirayama, T., Fukamachi, H. and Noda, M. (2003). Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. *Nat. Genet*. **33,** 375–381.

Fujita, K., Katahira, J., Horiguchi, Y., Sonoda, N., Furuse, M. and Tsukita, S. (2000). *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Lett*. **476,** 258–261.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S. (1998). Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol*. **141,** 1539–1550.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R. and Haas, R. (2003). *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301,** 1099–1102.

Gill, D.M. and Pappenheimer, A.M., Jr. (1971). Structure-activity relationships in diphtheria toxin. *J. Biol. Chem*. **246,** 1492–1495.

Goishi, K., Higashiyama, S., Klagsbrun, M., Nakano, N., Umata, T., Ishikawa, M., Mekada, E. and Taniguchi, N. (1995). Phorbol ester induces the rapid processing of cell surface heparin-binding, EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6,** 967–980.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of Bordetella pertussis binds to target cells via the $a_M b_2$ integrin (CD11b/CD18). *J. Exp. Med*. **193,** 1035–1044.

Hanna, P.C., Mietzner, T.A., Schoolnik, G.K. and McClane, B.A. (1991). Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. *J. Biol. Chem*. **266,** 11037–11043.

Higashiyama, S., Abraham, J.A. and Klagsbrun, M. (1993). Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J. Cell. Biol*. **122,** 933–940.

Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251,** 936–939.

Hollenberg, M.D., Fishman, P.H., Bennett, V. and Cuatrecasas, P. (1974). Cholera toxin and cell growth: role of membrane gangliosides. *Proc. Natl. Acad. Sci. USA* **71,** 4224–4228.

Holmgren, J. (1973). Comparison of the tissue receptors for *Vibrio cholerae* and Escherichia coli enterotoxins by means of gangliosides and natural cholera toxoid. *Infect. Immun*. **8,** 851–859.

Holmgren, J., Lonnroth, I., Mansson, J. and Svennerholm, L. (1975). Interaction of cholera toxin and membrane GM1 ganglioside of small intestine. *Proc. Natl. Acad. Sci. USA* **72,** 2520–2524.

Honjo, T., Nishizuka, Y., Hayaishi, O. and Kato, I. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem*. **243,** 3553–3555.

Horiguchi, Y., Akai, T. and Sakaguchi, G. (1987). Isolation and function of a *Clostridium perfringens* enterotoxin fragment. *Infect. Immun*. **55,** 2912–2915.

Horiguchi, Y., Uemura, T., Kozaki, S. and Sakaguchi, G. (1985). The relationship between cytotoxic effect and binding to mammalian cultured cells of *Clostridium perfringens* enterotoxin. *FEMS Microbiol. Lett*. **28,** 131–135.

Hughes, J.M., Murad, F., Chang, B. and Guerrant, R.L. (1978). Role of cyclic GMP in the action of heat-stable enterotoxin of Escherichia coli. *Nature* **271,** 755–756.

Itelson, T.R. and Gill, D.M. (1973). Diphtheria toxin: Specific competition for cell receptors. *Nature* **242,** 330–332.

Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1994). Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO J*. **13,** 2322–2330.

Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G., Nanba, D., Higashiyama, S., Hori, M., Klagsbrun, M. and Mekada, E. (2003). Heparin-binding, EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. USA* **100,** 3221–3226.

Izumi, Y., Hirata, M., Hasuwa, H., Iwamoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S. and Mekada, E. (1998). A metalloprotease-disintegrin, MDC9/Meltrin-g/ADAM9, and PKCd are involved in TPA-induced ectodomain shedding of membrane-anchored, heparin-binding EGF-like growth factor. *EMBO J*. **17,** 7260–7272.

Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolfe, A. and Keusch, G.T. (1986). Pathogenesis of shigella diarrhea. XI. Isolation of a shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J. Exp. Med*. **163,** 1391–1404.

Kamata, Y., Kozaki, S., Sakaguchi, G., Iwamori, M. and Nagai, Y. (1986). Evidence for direct binding of *Clostridim botulinum* type E derivative toxin and its fragments to gangliosides and free fatty acids. *Biochem. Biophys. Res. Commun*. **140,** 1015–1019.

Katahira, J., Inoue, N., Horiguchi, Y., Matsuda, M. T. and Sugimoto, N. (1997a). Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. *J. Cell Biol*. **136,** 1239–1247.

Katahira, J., Sugiyama, H., Inoue, N., Horiguchi, Y., Matsuda, M. and Sugimoto, N. (1997b). *Clostridium perfringens* enterotoxin utilizes two structurally related membrane proteins as functional receptors *in vivo*. *J. Biol. Chem*. **272,** 26652–26658.

Keusch, G.T. and Jacewicz, M. (1977). Pathogenesis of Shigella diarrhea. VII. Evidence for a cell membrane toxin receptor involving beta1 leads to 4-linked N-acetyl-D-glucosamine oligomers. *J. Exp. Med*. **146,** 535–546.

Kitamura, M., Iwamori, M. and Nagai, Y. (1979). Interaction between *Clostridium botulinum* neurotoxin and gangliosides. *Biochim. Biophys. Acta* **628,** 328–335.

Kokai-Kun, J.F., Benton, M., Wieckowski, E.U. and McClane, B.A. (1999). Identification of a Clostridium perfringens enterotoxin region required for large complex formation and cytotoxicity by random mutagenesis. *Infect. Immun*. **67,** 5634–5641.

Kounnas, M.Z., Morris, R.E., Thompson, M.R., FitzGerald, D.J., Strickland, D.K. and Saelinger, C.B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem*. **267,** 12420–12423.

Kozaki, S. (1979). Interaction of botulinum type A, B, and E derivative toxins with synaptosomes of rat brain. *Naunyn-Schmiedeberg's Arch. Pharmacol*. **308,** 67–70.

Kozaki, S., Kamata, Y., Watarai, S., Nishiki, T. and Mochida, S. (1998). Ganglioside GT1b as a complementary receptor component for Clostridium botulinum neurotoxins. *Microb. Pathog*. **25,** 91–99.

Kozaki, S., Miki, A., Kamata, Y., Ogasawara, J. and Sakaguchi, G. (1989). Immunological characterization of papain-induced fragments of *Clostridium botulinum* type A neurotoxin and interaction of the fragments with brain synaptosomes. *Infect. Immun*. **57,** 2634–2639.

Kozaki, S., Sakaguchi, G., Nishimura, M., Iwamori, M. and Nagai, Y. (1984). Inhibitory effect of ganglioside $GT_{1b}$ on the activities of *Clostridium botulinum* toxins. *FEMS Microbiol. Lett*. **21,** 219–223.

Lacy, D.B., Wigelsworth, D.J., Melnyk, R.A., Harrison, S.C. and Collier, R.J. (2004a). Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. *Proc. Natl. Acad. Sci. USA* **101,** 13147–13151.

Lacy, D.B., Wigelsworth, D.J., Scobie, H.M., Young, J.A. and Collier, R.J. (2004b). Crystal structure of the von Willebrand factor A domain of human capillary morphogenesis protein 2: an anthrax toxin receptor. *Proc. Natl. Acad. Sci. USA* **101,** 6367–6372.

Lemjabbar, H. and Basbaum, C. (2002). Platelet-activating factor receptor and ADAM10 mediate responses to Staphylococcus aureus in epithelial cells. *Nat. Med*. **8,** 41–46.

Leppla, S.H. (1995). Anthrax toxins. In: Bacterial Toxins and Virulence Factors in Disease, vol. 8, (eds. J. Moss, B. Iglewski, M. Vaughan and A.T. Tu) pp. 543–572 Marcel. Dekker, Inc., New York.

Louie, G.V., Yang, W., Bowman, M.E. and Choe, S. (1997). Crystal structure of the complex of diphtheria toxin with an extracellular fragment of its receptor. *Mol. Cell* **1,** 67–78.

Matteoli, M., Takei, K., Perin, M.S., Sudhof, T.C. and De Camilli, P. (1992). Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell. Biol*. **117,** 849–861.

McDonel, J.L. (1980). Binding of *Clostridium perfringens* ($^{125}$I)enterotoxin to rabbit intestinal cells. *Biochemistry* **19,** 4801–4807.

McDonel, J.L. and McClane, B.A. (1979). Binding versus biological activity of *Clostridium perfringens* enterotoxin in Vero cells. *Biochem. Biophys. Res. Commun*. **87,** 497–504.

Mekada, E., Okada, Y. and Uchida, T. (1988). Identification of diphtheria toxin receptor and a nonproteinous diphtheria toxin-binding molecule in Vero cell membrane. *J. Cell Biol*. **107,** 511–519.

Mekada, E., Senoh, H., Iwamoto, R., Okada, Y. and Uchida, T. (1991). Purification of diphtheria toxin receptor from Vero cells. *J. Biol. Chem*. **266,** 20457–20462.

Merritt, E.A., Kuhn, P., Sarfaty, S., Erbe, J.L., Holmes, R.K. and Hol, W.G. (1998). The 1.25 A resolution refinement of the cholera toxin B-pentamer: evidence of peptide backbone strain at the receptor-binding site. *J. Mol. Biol*. **282,** 1043–1059.

Merritt, E.A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J.A. and Hol, W.G. (1994). Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci*. **3,** 166–175.

Middlebrook, J.L. and Dorland, R.B. (1977). Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*:differential cytotoxicity. *Can. J. Microbiol*. **23,** 183–189.

Middlebrook, J.L., Dorland, R.B. and Leppla, S.H. (1978). Association of diphtheria toxin with Vero cells. *J. Biol. Chem*. **253,** 7325–7330.

Mitamura, T., Higashiyama, S., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding, EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem*. **270,** 1015–1019.

Mitamura, T., Iwamoto, R., Umata, T., Yomo, T., Urabe, I., Tsuneoka, M. and Mekada, E. (1992). The 27-kD diphtheria toxin receptor-associated protein (DRAP27) from Vero cells is the monkey homolog of human CD9 antigen: expression of DRAP27 elevates the number of diphtheria toxin receptors on toxin-sensitive cells. *J. Cell Biol*. **118,** 1389–1399.

Mitamura, T., Umata, T., Nakano, F., Shishido, Y., Toyoda, T., Itai, A., Kimura, H. and Mekada, E. (1997). Structure-function analysis of the diphtheria toxin receptor toxin binding site by site-directed mutagenesis. *J. Biol. Chem*. **272,** 27084–27090.

Miyagawa, J., Higashiyama, S., Kawata, S., Inui, Y., Tamura, S., Yamamoto, K., Nishida, M., Nakamura, T., Yamashita, S., Matsuzawa, Y. *et al*. (1995). Localization of heparin-binding, EGF-like growth factor in the smooth muscle cells and macrophages of human atherosclerotic plaques. *J. Clin. Invest*. **95,** 404–411.

Miyamoto, S., Hirata, M., Yamazaki, A., Kageyama, T., Hasuwa, H., Mizushima, H., Tanaka, Y., Yagi, H., Sonoda, K., Kai, M., Kanoh, H., Nakano, H. and Mekada, E. (2004). Heparin-binding, EGF-like growth factor is a promising target for ovarian cancer therapy. *Cancer Res*. **64,** 5720–5727.

Molloy, S.S., Bresnahan, P.A., Leppla, S.H., Klimpel, K.R. and Thomas, G. (1992). Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J. Biol. Chem*. **267,** 16396–16402.

Morita, K., Furuse, M., Fujimoto, K. and Tsukita, S. (1999a). Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc. Natl. Acad. Sci. USA* **96,** 511–516.

Morita, K., Sasaki, H., Fujimoto, K., Furuse, M. and Tsukita, S. (1999b). Claudin-11/OSP-based tight junctions of myelin sheaths in brain and sertoli cells in testis. *J. Cell Biol*. **145,** 579–588.

Moss, J., Fishman, P.H., Manganiello, V.C., Vaughan, M. and Brady, R.O. (1976). Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. *Proc. Natl. Acad. Sci. USA* **73,** 1034–1037.

Naglich, J.G. and Eidels, L. (1990). Isolation of diphtheria toxin-sensitive mouse cells from a toxin-resistant population transfected with monkey DNA. *Proc. Natl. Acad. Sci. USA* **87,** 7250–7254.

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: Identity with a heparin-binding, EGF-like growth factor precursor. *Cell* **69,** 1051–1061.

Nakagawa, T., Higashiyama, S., Mitamura, T., Mekada, E. and Taniguchi, N. (1996). Amino-terminal processing of cell surface heparin-binding epidermal growth factor-like growth factor upregulates its juxtacrine but not its paracrine growth factor activity. *J. Biol. Chem*. **271,** 30858–30863.

Nelson, K.L., Raja, S.M. and Buckley, J.T. (1997). The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *J. Biol. Chem*. **272,** 12170–12174.

Nishiki, T., Kamata, Y., Nemoto, Y., Omori, A., Ito, T., Takahashi, M. and Kozaki, S. (1994). Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes. *J. Biol. Chem*. **269,** 10498–10503.

Nishiki, T., Ogasawara, J., Kamata, Y. and Kozaki, S. (1993). Solubilization and characterization of the acceptor for *Clostridium botulinum* type B neurotoxin from rat brain synaptic membranes. *Biochim. Biophys. Acta*. **1158,** 333–338.

Nishiki, T., Tokuyama, Y., Kamata, Y., Nemoto, Y., Yoshida, A., Sato, K., Sekiguchi, M., Takahashi, M. and Kozaki, S. (1996a). The high-affinity binding of Clostridium botulinum type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1a. *FEBS Lett*. **378,** 253–257.

Nishiki, T., Tokuyama, Y., Kamata, Y., Nemoto, Y., Yoshida, A., Sekiguchi, M., Takahashi, M. and Kozaki, S. (1996b). Binding of botulinum type B neurotoxin to Chinese hamster ovary cells transfected with rat synaptotagmin II cDNA. *Neurosci. Lett*. **208,** 105–108.

Padilla, P.I., Wada, A., Yahiro, K., Kimura, M., Niidome, T., Aoyagi, H., Kumatori, A., Anami, M., Hayashi, T., Fujisawa, J., Saito, H.,

Moss, J. and Hirayama, T. (2000). Morphologic differentiation of HL-60 cells is associated with appearance of RPTPβ and induction of *Helicobacter pylori* VacA sensitivity. *J. Biol. Chem*. **275,** 15200–15206.

Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X., Burroni, D., Cover, T.L., Papini, E., Rappuoli, R., Telford, J.L. and Reyrat, J. (1998). The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc. Natl. Acad. Sci. USA* **95,** 10212–10217.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Raab, G. and Klagsbrun, M. (1997). Heparin-binding, EGF-like growth factor. *Biochim. Biophys. Acta*. **1333,** F179–199.

Santelli, E., Bankston, L.A., Leppla, S.H. and Liddington, R.C. (2004). Crystal structure of a complex between anthrax toxin and its host cell receptor. *Nature* **430,** 905–908.

Schulz, S., Green, C.K., Yuen, P.S. and Garbers, D.L. (1990). Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* **63,** 941–948.

Scobie, H.M., Rainey, G.J., Bradley, K.A. and Young, J.A. (2003). Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc. Natl. Acad. Sci. USA* **100,** 5170–5174.

Shishido, Y., Sharma, K.D., Higashiyama, S., Klagsbrun, M. and Mekada, E. (1995). Heparin-like molecules on the cell surface potentiate binding of diphtheria toxin to the diphtheria toxin receptor/membrane-anchored heparin-binding epidermal growth factor-like growth factor. *J. Biol. Chem*. **270,** 29578–29585.

Simpson, L.L. and Rapport, M.M. (1971). The binding of botulinum toxin to membrane lipids: sphingolipids and ffatty acids. *J. Neurochem*. **18,** 1751–1759.

Singh, U., Van Itallie, C.M., Mitic, L.L., Anderson, J.M. and McClane, B.A. (2000). CaCo-2 cells treated with Clostridium perfringens enterotoxin form multiple large complex species, one of which contains the tight junction protein occludin. *J. Biol. Chem*. **275,** 18407–18417.

Singh, Y., Klimpel, K.R., Quinn, C.P., Chaudhary, V.K. and Leppla, S.H. (1991). The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *J. Biol. Chem*. **266,** 15493–15497.

Sonoda, N., Furuse, M., Sasaki, H., Yonemura, S., Katahira, J., Horiguchi, Y. and Tsukita, S. (1999). *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol*. **147,** 195–204.

Sugii, S. and Horiguchi, Y. (1988). Identification and isolation of the binding substance for *Clostridium perfringens* enterotoxin on Vero cells. *FEMS Microbiol. Lett*. **52,** 85–90.

Sundrud, M.S., Torres, V.J., Unutmaz, D. and Cover, T.L. (2004). Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc. Natl. Acad. Sci. USA* **101,** 7727–7732.

Takazaki, R., Shishido, Y., Iwamoto, R. and Mekada, E. (2004). Suppression of the biological activities of the epidermal growth factor (EGF)-like domain by the heparin-binding domain of heparin-binding EGF-like growth factor. *J. Biol. Chem*. **279,** 47335–47343.

Takemura, T., Hino, S., Kuwajima, H., Yanagida, H., Okada, M., Nagata, M., Sasaki, S., Barasch, J., Harris, R.C. and Yoshioka, K. (2001). Induction of collecting duct morphogenesis *in vitro* by heparin-binding epidermal growth factor-like growth factor. *J. Am. Soc. Nephrol*. **12,** 964–972.

Takenobu, H., Yamazaki, A., Hirata, M., Umata, T. and Mekada, E. (2003). The stress- and inflammatory cytokine-induced ectodomain shedding of heparin-binding epidermal growth factor-like growth factor is mediated by p38 MAPK, distinct from the 12-O-tetradecanoylphorbol-13-acetate- and lysophosphatidic acid-induced signaling cascades. *J. Biol. Chem*. **278,** 17255–17262.

Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J.I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y., Matsuzawa, Y., Hashimoto, K. and Taniguchi, N. (2000). Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell. Biol*. **151,** 209–220.

Thompson, M.R., Forristal, J., Kauffmann, P., Madden, T., Kozak, K., Morris, R.E. and Saelinger, C.B. (1991). Isolation and characterization of Pseudomonas aeruginosa exotoxin A binding glycoprotein from mouse LM cells. *J. Biol. Chem*. **266,** 2390–2396.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell. Biol*. **2,** 285–293.

Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N. and Mekada, E. (1993). Evidence for involvement of furin in cleavage and activation of diphtheria toxin. *J. Biol. Chem*. **268,** 26461–26465.

Uchida, T., Pappenheimer, A.M., Jr. and Harper, A.A. (1972). Reconstitution of diphtheria toxin from two nontoxic cross-reacting mutant proteins. *Science* **175,** 901–903.

Umata, T., Hirata, M., Takahashi, T., Ryu, F., Shida, S., Takahashi, Y., Tsuneoka, M., Miura, Y., Masuda, M., Horiguchi, Y. and Mekada, E. (2001). A dual signaling cascade that regulates the ectodomain shedding of heparin-binding epidermal growth factor-like growth factor. *J. Biol. Chem*. **276,** 30475–30482.

Umata, T., Sharma, K.D. and Mekada, E. (eds.) (2000). Diphtheria toxin and the diphtheria-toxin receptor. Springer-Verlag, Heidelberg.

van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F. and Greenough III, W.B. (1971). Deactivation of cholera toxin by ganglioside. *J. Infect. Dis*. **124,** 415–418.

Waldman, S.A., Kuno, T., Kamisaki, Y., Chang, L.Y., Gariepy, J., O'Hanley, P., Schoolnik, G. and Murad, F. (1986). Intestinal receptor for heat-stable enterotoxin of Escherichia coli is tightly coupled to a novel form of particulate guanylate cyclase. *Infect. Immun*. **51,** 320–326.

Wieckowski, E.U., Wnek, A.P. and McClane, B.A. (1994). Evidence that an ≈50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. *J. Biol. Chem*. **269,** 10838–10848.

Wnek, A.P. and McClane, B.A. (1983). Identification of a 50,000 Mr protein from rabbit brush border membranes that binds *Clostridium perfringens* enterotoxin. *Biochem. Biophyss. Res. Commun*. **112,** 1099–1105.

Wnek, A.P. and McClane, B.A. (1986). Comparison of receptors for *Clostridium perfringens* type A and cholera enterotoxins in isolated rabbit intestinal brush border membranes. *Microb. Pathogen*. **1,** 89–100.

Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P.I., Wada, A. and Hirayama, T. (1997). Helicobacter pylori vacuolating cytotoxin binds to the 140-kDa protein in human gastric cancer cell lines, AZ-521 and AGS. *Biochem. Biophys. Res. Com*. **238,** 629–632.

Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J. and Hirayama, T. (1999). Activation of Helicobacter pylori VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase beta. *J. Biol. Chem*. **274,** 36693–36699.

Yahiro, K., Wada, A., Nakayama, M., Kimura, T., Ogushi, K., Niidome, T., Aoyagi, H., Yoshino, K., Yonezawa, K., Moss, J. and Hirayama, T. (2003). Protein-tyrosine phosphatase α, RPTPα, is a *Helicobacter pylori* VacA receptor. *J. Biol. Chem*. **278,** 19183–19189.

Yahiro, K., Wada, A., Yamasaki, E., Nakayama, M., Nishi, Y., Hisatsune, J., Morinaga, N., Sap, J., Noda, M., Moss, J. and Hirayama, T. (2004). Essential domain of receptor tyrosine phosphatase β (RPTPβ) for interaction with *Helicobacter pylori* vacuolating cytotoxin. *J. Biol. Chem*. **279,** 51013–51021.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15,** 245–250.

Yamazaki, S., Iwamoto, R., Saeki, K., Asakura, M., Takashima, S., Yamazaki, A., Kimura, R., Mizushima, H., Moribe, H., Higashiyama, S., Endoh, M., Kaneda, Y., Takagi, S., Itami, S., Takeda, N., Yamada, G. and Mekada, E. (2003). Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J. Cell. Biol*. **163,** 469–475.

Zhang, D., Takahashi, J., Seno, T., Tani, Y. and Honda, T. (1999). Analysis of receptor for *Vibrio cholerae* El tor hemolysin with a monoclonal antibody that recognizes glycophorin B of human erythrocyte membrane. *Infect. Immun*. **67,** 5332–5337.

CHAPTER

# 7

# Translocation of bacterial protein toxins into the cytosol

*Sjur Olsnes and Jørgen Wesche*

## INTRODUCTION AND BRIEF DESCRIPTION OF RELEVANT TOXINS

Bacterial protein toxins act either at the level of the cell surface or at targets in the cytosol. Superantigens and toxins containing lytic activity exert their action at the level of the cell surface and will not be discussed here. However, an increasing number of bacterial protein toxins are being demonstrated to have intracellular sites of action. This implies that the toxins must be able to cross cellular membranes. Most toxins do this by crossing membranes of intracellular organelles rather than penetrating through the plasma membrane.

Some toxins are equipped with their own translocation apparatus, whereas others must rely on cellular translocation processes that they exploit. This is most clearly evident in the case of immunotoxins. These constructs consist of an antibody that binds to target cells and a protein that is highly toxic once in the cytosol, but that is unable to translocate to the cytosol on its own. Recent research has shed some light on how these toxins and immunotoxins enter the cytosol. The toxins that will be particularly considered here are depicted schematically in Figure 7.1.

Diphtheria toxin is synthesized as a single chain protein with a signal sequence that is cleaved off when the toxin is excreted from the bacteria. The mature toxin is easily cleaved into two fragments by trypsin-like proteases, and the cleaved toxin represents the active form. It consists of an enzymatically active part, the A-fragment, which is linked by a disulfide bond to the B-fragment (Pappenheimer, Jr., 1977). The B-fragment consists of a receptor-binding domain (R) and a transmembrane domain (T), which can insert into the membrane and which plays a decisive role in the translocation of the A-fragment to the cytosol (Choe *et al.*, 1992). It resembles membrane-inserting domains in certain colicins and in the δ-toxin from *Bacillus thuringensis* (Li *et al.*, 1991). Once in the cytosol the A-fragment ADP-ribosylates a unique amino acid, diphthamide, in elongation factor 2, thereby inactivating the elongation factor and inhibiting protein synthesis (Collier, 1975; Van Ness *et al.*, 1980a; Van Ness *et al.*, 1980b).

*Pseudomonas aeruginosa* exotoxin A resembles in many respects diphtheria toxin. It has an enzymatically active part (domain III) that has the same activity as diphtheria toxin A-fragment and a region (domain II) that structurally reminds of the T-domain of diphtheria toxin and that is therefore believed to be involved in the translocation process (Wick *et al.*, 1990). It differs from diphtheria toxin and most other toxins in this group in that the enzymatically active domain of exotoxin A is located in the C-terminal part of the molecule. A disulfide-bridged cleavage site sensitive to furin is located in domain II, and it is believed that cleavage and reduction is required for translocation (Allured *et al.*, 1986). Domain I binds the toxin to cell surface receptors. The C-terminus of the toxin has a KDEL-like structure, suggesting that the toxin is transported to the endoplasmic reticulum, where it may be translocated to the cytosol.

The A–$B_5$ class of toxins comprises cholera toxin, *E. coli* heat-labile toxin, Shiga toxin, and Shiga-like toxins 1 and 2 (Merritt and Hol, 1995; Lencer and Tsai,

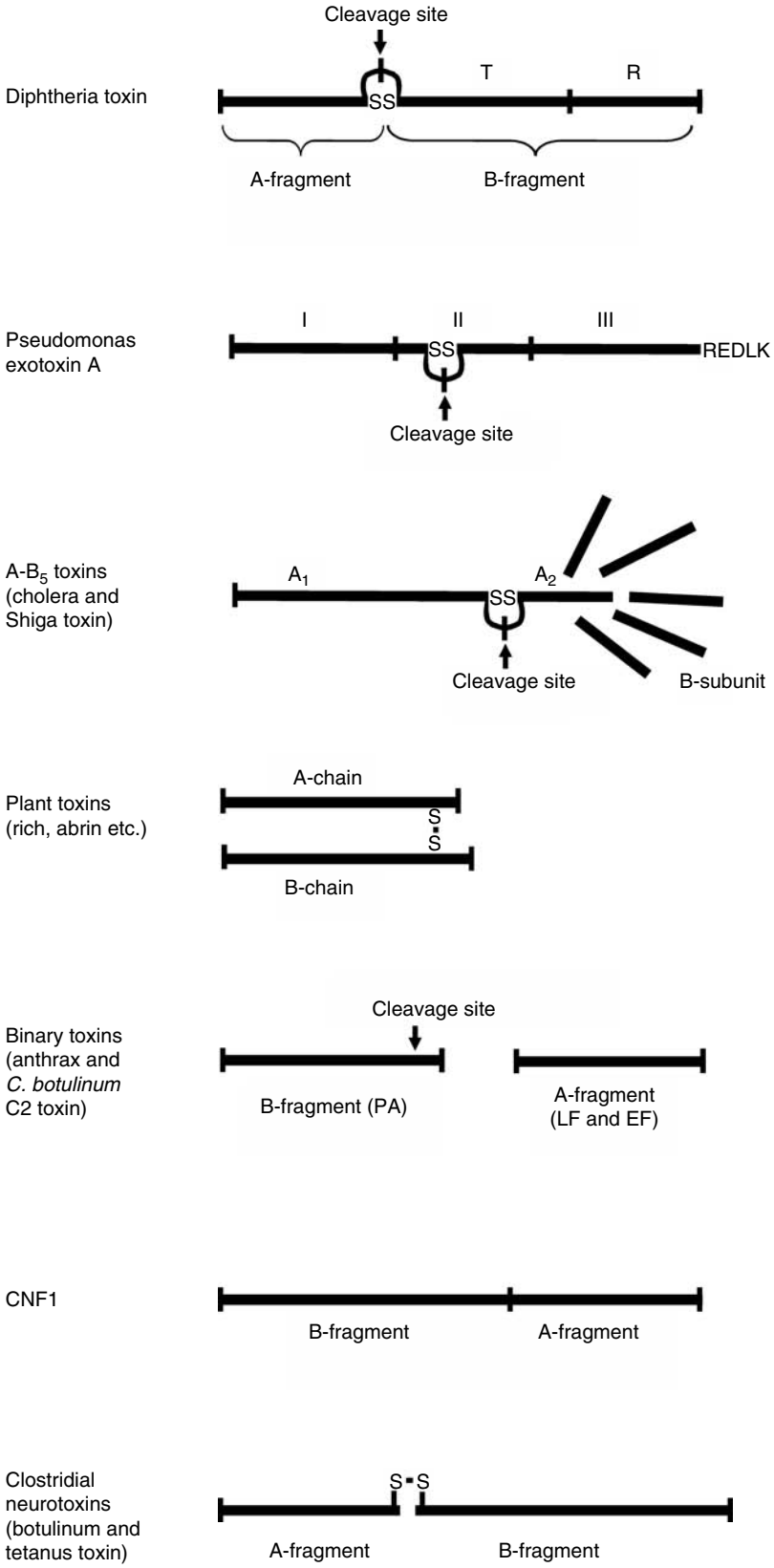



**FIGURE 7.1** Schematic structure of toxins. Details are discussed in the text.

2003). These toxins have a similar overall structure, but they have different functions. The toxins all consist of a pentameric B-subunit that is linked non-covalently to the C-terminal part of the A-subunit, which consists of a single polypeptide chain. The A-subunit contains in its C-terminal end a disulfide-bridged trypsin- and furin-sensitive loop, which is easily cleaved to yield the enzymatically active $A_1$-fragment and the small $A_2$-fragment that is associated with the B-subunit (Olsnes *et al*., 1981; Reisbig *et al*., 1981). The B-subunit of all toxins in this group binds to glycolipids. The enzymatically active A-subunits of cholera toxin and heat-labile *E. coli* toxin ADP-ribosylate and permanently activate the α-subunit of trimeric G-proteins, whereas the A-subunit of Shiga toxin and Shiga-like toxins inactivate ribosomes by removal of an adenine residue from the 28S RNA of the large ribosomal subunit, thereby inhibiting protein synthesis.

We will also discuss a group of plant toxins comprising ricin, abrin, modeccin, volkensin, and others that have properties similar to the bacterial toxins we are considering here (Olsnes *et al*., 1974). These toxins are synthesized as a continuous polypeptide chain, but prior to excretion they are cleaved in the vacuole of the plant cells to yield an enzymatically active A-chain and a receptor-binding B-chain held together by a disulfide bond (Figure 7.1). The B-chain has lectin properties and binds to surface glycoproteins and glycolipids with terminal galactose residues (Sandvig and Olsnes, 1979; Olsnes and Refsnes, 1978). The A-chain has the same enzymatic activity as the A-subunit of Shiga toxin (Endo *et al*., 1988).

The binary toxins are produced as two different proteins, one that binds to receptors at the cell's surface, and which is then cleaved to release a ~20 kDa fragment. In the case of anthrax toxin, and probably also other binary toxins, the remaining major part of the cleaved molecules forms heptamers that act as binding sites for an enzymatically active protein that is able to enter the cytosol. The *Clostridium botulinum* C2 toxin, *C. perfringens iota*-toxin, and *C. spiriforme* toxin are binary toxins that act by ADP-ribosylating the ATP-binding protein, actin. There are at least six mammalian actin isoforms, but *C. botulinum* C2 toxin ADP-ribosylates only the cytoplasmic form (Aktories *et al*., 1992; Aktories *et al*., 1986). Anthrax toxin is most studied among the binary toxins. In this case two different enzymatically active proteins, the edema factor and the lethal factor, can use the same binding subunit (called *PA* or *protective antigen*) to which they bind in a mutually exclusive manner. The lethal factor is a metalloprotease that cleaves MAP kinase kinase (Duesbery *et al*., 1998; Vitale *et al*., 1998). The edema factor is an adenylate cyclase that enters the cytosol where it is activated by calmodulin (Leppla, 1982), leading to elevation of the level of cAMP, thus interfering with the bacteriocidal capacity of the intoxicated cell.

Another invasive and calmodulin-activated adenylate cyclase (CyaA) is produced by *Bordetella pertussis* (Rogel *et al*., 1991). The 177 kD CyaA-protein can be divided into an N-terminal catalytic domain and a C-terminal hemolytic domain. Upon activation by calmodulin, CyaA elevates the level of cAMP in the cytosol.

An export protein from *Clostridium botulinum*, exoenzyme C3, is able to ADP-ribosylate the small G-protein Rho, which regulates actin polymerization and intracellular vesicular transport (Chardin *et al*., 1989). Similar enzymes are found in a wide variety of bacteria (Just *et al*., 1992a; Just *et al*., 1992b; Lencer *et al*., 2003) including *Staphylococcus aureus* (Inoue *et al*., 1991), although no bacterial substrate is known. Use of exoenzyme C3 has played an important role in the recent elucidation of the mechanism of action of certain microbial toxins, the *Clostridium difficile* toxins A and B (Just *et al*., 1995a) and the *E. coli* necrotizing toxin (CNF1) (Lemichez *et al*., 1997). When Rho had been pretreated with *C. difficile* toxins, it could no longer be ADP-ribosylated by exoenzyme C3, clearly indicating that the *C. difficile* toxin had modified Rho. This modification was later found to consist in transfer of glucose from the precursor UDP-Glc to a threonine residue in Rho and the related Rac and Cdc 42 (Just *et al*., 1995a; Just *et al*., 1995b). Lethal toxin from *C. sordellii* has the same activity, which leads to the disassembly of stress fibers in the toxin-treated cells (Popoff *et al*., 1996; Selzer *et al*., 1996). The toxins did not modify the more distantly related G-proteins ras, rab, and ARF. *C. novyi* toxin incorporates N-acetylglucosamine rather than glucose into rho (Just *et al*., 1996).

Cytotoxic necrotizing toxin (CNF1) from certain strains of *E. coli* is another toxin acting on Rho. The toxin consists of a single polypeptide chain (137 kD) with a putative N-terminal receptor-binding domain, a central hydrophobic domain, and a C-terminal catalytic domain (Lemichez *et al*., 1997). The toxin induces deamidation of a glutamine residue (Gln63) (Flatau *et al*., 1997; Schmidt *et al*., 1997), leading to permanent activation of Rho. As a result, there is a strong overactivity in actin polymerization and formation of multinucleated cells. Furthermore, the toxin induces phagocytosis in non-phagocytic cells, enabling toxigenic bacteria to enter and multiply in cells and to be transcytosed across epithelia. It is apparently part of the same strategy that the toxin inhibits apoptosis in the intoxicated cells (Fiorentini *et al*., 1997).

The neurotoxins tetanus toxin and botulinum toxins are not produced as export proteins, but rather as intracellular proteins that are released when the bacterium dies. The molecular action of these toxins has been elucidated in great detail in recent years. They are all metalloproteases that cleave proteins involved in exocytosis, particularly in the synapses (Schiavo *et al*., 1992; Montecucco and Schiavo, 1993). As a result, the synaptic transmission is paralyzed. Also, these toxins are formed as a single polypeptide chain, which is cleaved into an enzymatically active fragment and a fragment that is involved in binding and translocation. As in the case of diphtheria toxin, the two parts are disulfide-linked. Binding to different surface receptors is probably the reason that different neurons are affected in tetanus and botulism.

*Helicobacter pylori* produces a vacuolating toxin,VacA, which appears to be involved in the appearance of stomach ulcers. There are now clear indications that this toxin enters the cytosol. Thus, the vacuolation can be induced by intracellular expression of the toxin (Papini *et al*., 2001), it interacts with the intermediary filament protein, vimentin (de Bernard *et al*., 2000), and a 37 kD fragment of the toxin enters the mitochondria and induces release of cytochrome C and apoptosis of the cell (Boquet *et al*., 2003).

## BINDING TO CELL SURFACE RECEPTORS

All bacterial protein toxins must bind to cells in order to act. Since for obvious reasons the cells have not developed receptors for toxins, the toxins must employ cell surface structures that are designed for other purposes. Cells lacking the relevant receptors are resistant to the toxin. The surface molecules that the toxins use for binding may be hormone receptors, growth factor precursors, cell adhesion molecules, glycolipids, and other molecules. So far, there has not emerged any particular property that is common for toxin receptors. However, it is likely that the ability to transport the toxin to defined intracellular compartments will prove to be a common property.

Diphtheria toxin binds to the uncleaved precursor of a growth factor, namely that of heparin-binding, EGF-like growth factor (HB-EGF) (Naglich *et al*., 1992). All growth factors of the EGF family are formed as transmembrane proteins that are transported out of the cells and then cleaved to release the growth factor. Not all molecules are cleaved, and it is now clear that in many cases the uncleaved molecules act as growth factors in a juxtacrine manner by binding to EGF-receptors on adjacent cells (Raab and Klagsbrun, 1997; Takemura *et al*., 1997). Diphtheria toxin binds to the HB-EGF precursor present on most mammalian cells. Due to a few amino acids' difference, the HB-EGF precursor found in mouse and rat cells does not bind to the toxin, and cells from these animals are therefore resistant to diphtheria toxin (Hooper and Eidels, 1996). Transgenic mice expressing HB-EGF precursor from monkeys became sensitive to the toxin (Cha *et al*., 2003). By placing the gene behind an organ-specific promoter, selective deletion of the corresponding organ could be induced by intramuscular administration of diphtheria toxin (Saito *et al*., 2001; Akazawa *et al*., 2004).

The correct distance of the receptor-bound toxin to the membrane seems to be essential. Thus, when the distance was slightly increased by extending the juxtamembrane domain of the receptor by a few amino acids, it strongly reduced the ability of the receptor to facilitate translocation of A-fragment to the cytosol (Takahashi *et al*., 2001) in accordance with earlier studies (Lanzrein *et al*., 1996).

Two closely related cell adhesion molecules were recently shown to act as receptors for anthrax toxin (Bradley *et al*., 2001; Lacy *et al*., 2004; Scobie *et al*., 2003). Like the diphtheria toxin receptor, they are transmembrane proteins that pass the membrane once. Most of the extracellular part consists of a von Willebrand factor A domain that binds the protective antigen (PA) of the toxin. The affinity is very high in the high picomolar range and involves a $Mg^{2+}$-ion and a MIDAS motif (Lacy *et al*., 2004; Santelli *et al*., 2004). The structure of PA bound to the receptor has been solved (Lacy *et al*., 2004; Santelli *et al*., 2004; Bann and Hultgren, 2004).

Pseudomonas exotoxin A binds to a cell surface protein that binds to low-density lipoprotein and $\alpha_2$-macroglobulin (Kounnas *et al*., 1992).

Cholera toxin and *E. coli* heat-labile toxin bind to the ganglioside $Gm_1$ (van Heyningen, 1974), whereas Shiga toxin binds to neutral glycolipids. Shigella (or Shiga) toxin, the essentially identical Shiga-like toxin 1, and the slightly different Shiga-like toxin 2 bind to globoside $B_3$ (Ling *et al*., 1998), whereas the related Shiga-like 2e toxin binds to globoside $B_4$ (DeGrandis *et al*., 1989).

One of the botulinum toxins, toxin B, binds to the neuronal protein synaptotagmin (Nishiki *et al*., 1994). The receptors for the other botulinum toxins have not been identified.

The plant toxins ricin, abrin, and related toxins are rather unselective as they bind both to glycoproteins and glycolipids with terminal galactose residues (Meager *et al*., 1976; Hughes and Gardas, 1976; Olsnes *et al*., 1978). Therefore, it is not clear which molecules are the productive receptors as it is likely that

binding to most of the structures carrying terminal galactose will not result in translocation of the A-chain to the cytosol.

The *Helicobacter pylori* toxin, VacA, appears to bind to a GPI-anchored protein at the cell surface and is endocytosed by a mechanism not involving clathrin (Ricci *et al*., 2000).

The nature of the receptors for most protein toxins that enter the cytosol is not known.

## ENDOCYTOSIS

Since most toxins that act on components in the cytosol are translocated from intracellular organelles, the uptake mechanism usually starts with endocytosis. The uptake may follow the classical route from clathrin coated pits, but in several cases it appears to occur from other surface structures. These may include caveoli or other, so far uncharacterized, invaginations of the plasma membrane.

The first indication that endocytosis may be necessary for entry of toxins came from experiments in reticulocytes with the plant toxins, ricin and abrin (Olsnes *et al*., 1974). The ribosomes of reticulocytes are very sensitive to abrin and ricin, but in spite of this, protein synthesis in whole reticulocytes was not inhibited after incubation with high concentrations of these toxins. Both toxins were found to bind extensively to the surface of the reticulocytes, but there was no evidence for endocytic uptake. The exception is the invasive adenylate cyclase from *B. pertussis* that appears normally to enter through the surface membrane (Rogel and Hanski, 1992).

More definitive evidence was obtained with diphtheria toxin in nucleated cells in culture. It was found that when the cells were incubated with ammonium chloride or other weak bases that are able to penetrate cellular membranes and neutralize acidic compartments in the cells, the cells were protected from intoxication (Kim and Groman, 1965). This protection was overcome when the cells were briefly exposed to low pH (Draper and Simon, 1980; Sandvig and Olsnes, 1980). Moreover, in the latter case, the cells were more rapidly intoxicated than when the cells were incubated with toxin under normal conditions. These results indicated that the normal entry pathway for the toxin is from intracellular acidic compartments, and that when cells with surface-bound toxin are exposed to low pH, the conditions in endosomes are mimicked at the level of the surface membrane, and direct translocation is induced.

Later, similar data have been obtained with anthrax toxin (Koehler and Collier, 1991; Milne and Collier, 1993; Wesche *et al*., 1998), *Cl. botulinum* C2 toxin (Barth *et al*., 2000), and CNF1 (Contamin *et al*., 2000). In the case of other toxins, it has not been possible to induce entry from the cell surface by treating cells with low pH or other conditions, and it is therefore likely that in this case the entry mechanism is more complicated.

Considerable additional evidence has accumulated that endocytosis is required for the toxins to enter the cytosol. Thus, when endocytosis was blocked by depleting the cells for ATP, the cells were not able to take up toxins (Sandvig and Olsnes, 1982b). Other experiments pointing in the same direction were carried out by depleting cells for calcium (Sandvig and Olsnes, 1982a) or exposing them to slightly reduced pH, conditions that do not interfere with endocytosis. Under these conditions, the cells are still able to endocytose the toxins, but the toxins are not able to intoxicate the cells. Treatment with antibodies to inactivate toxin remaining at the cell surface and in the medium was then followed by incubation of the cells in normal medium to relieve the block. Under these conditions, the cells were intoxicated by toxin endocytosed during the block.

At temperatures below 18°C endocytosis is still active, but transport to the trans-Golgi and to late endosomes is blocked. At this temperature the cells were not intoxicated by ricin. The cells were, however, fully sensitive to diphtheria toxin under these conditions (Sandvig *et al*., 1984). Evidence has accumulated over time that transport to the trans-Golgi network is the important routing for many toxins. Further experiments showed that brefeldin A and ilimaquinone that lead to disintegration of the Golgi apparatus, which is transported back to the ER, protect against ricin and Shigella toxin (Nambiar and Wu, 1995; Yoshida *et al*., 1991a; Rapak *et al*., 1997). These drugs did not affect the toxicity of diphtheria toxin. Clearly, therefore, there is a difference in the entry mechanism of the two groups of toxins (Figure 7.2). However, a main conclusion from these data is that in all cases endocytic uptake is a prerequisite for entry to the cytosol.

When the uptake of ricin by endocytosis from coated pits was blocked by depleting the cells for potassium, the cells were still intoxicated by ricin at approximately the same rate as in control cells. This provided the first evidence that toxins can enter the cytosol by mechanisms bypassing endocytosis from coated pits (Moya *et al*., 1985). Later experiments showed that when endocytic uptake from coated pits was inhibited by other mechanisms, such as acidification of the cytosol (but not of the medium) and by exposing the cells to hyperosmolaric medium, the cells were also intoxicated by ricin, but not by diphtheria toxin (Sandvig *et al*., 1989).

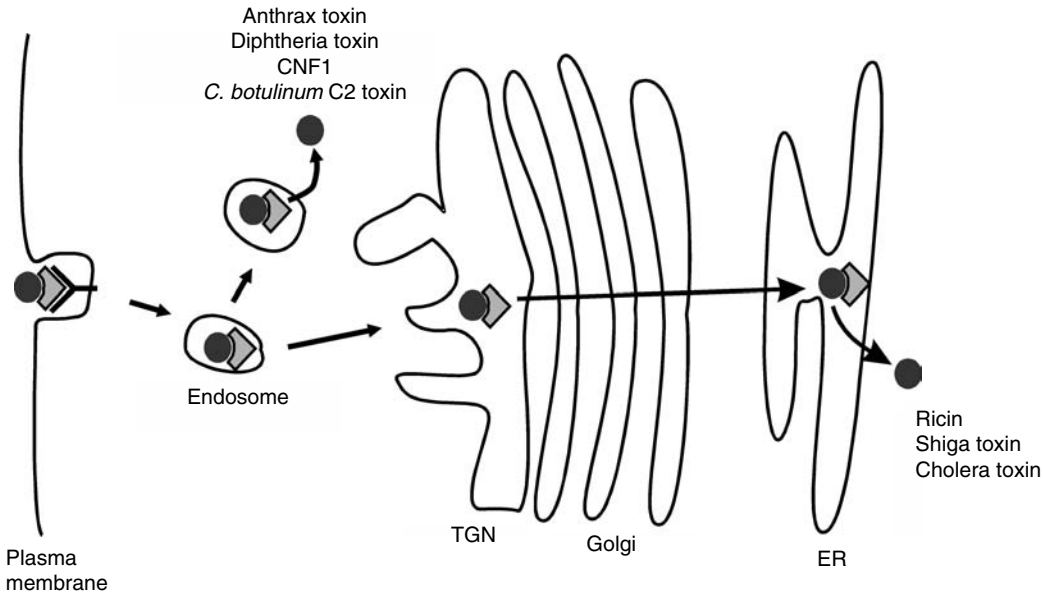



**FIGURE 7.2** Intracellular transport of toxins.

There is now considerable evidence that several toxins are endocytosed from structures different from the coated pits. It appears, however, that the toxin endocytosed by such an alternative pathway eventually ends up in the same endosomes as those taken up by the classical, clathrin-dependent pathway (van Deurs *et al*., 1989).

A more extensive description of endocytosis and vesicular transport of toxins is given in Chapter 8.

## RETROGRADE VESICULAR TRANSPORT

### Transport to the Golgi apparatus

As first observed with cholera toxin, a certain fraction of the endocytosed toxin is transported to the trans-Golgi network (Joseph *et al*., 1979). Later experiments demonstrated that this is also the case in a number of other toxins, such as Shiga toxin and ricin (Sandvig *et al*., 1992; Rapak *et al*., 1997). Furthermore, in the case of ricin it could be shown that the toxin is localized in the same compartment as newly synthesized export proteins. Thus, a cell line producing a monoclonal antibody against ricin was highly resistant to the toxin under conditions where the antibody was not able to inactivate the toxin at the cell exterior (Youle and Colombatti, 1987). More direct evidence was obtained in ultrastructural studies demonstrating that ricin imported from the medium met newly synthesized virus proteins in the trans-Golgi network (van Deurs *et al*., 1988). From here, it is then sorted to various locations in the cells, such as the endoplasmic reticulum, the lysosomes, or back to the cell surface. In polarized cells, the toxin may be transported to the opposite pole of the cells.

### Transport to the endoplasmic reticulum

A most interesting recent development in studies of toxin transport is the observation that a considerable part of the toxins that reach the Golgi apparatus is transported retrograde to the endoplasmic reticulum (ER) (Figure 7.2). The first suspicion that this may be the case came from structural observations with cholera toxin and the related *E. coli* heat-labile toxin. Both these toxins consist of a pentameric B-subunit that binds the toxins to gangliosides at the cell surface. The enzymatically active subunit, the A-subunit, contains in its C-terminal end the sequence KDEL (cholera toxin) and RDEL (heat-labile toxin). Since the only known function of this sequence is to bind to the KDEL receptor, it was suspected that this may indeed be involved in the intracellular routing of these two toxins (Chaudhary *et al*., 1990). Resident luminal ER proteins contain a C-terminal KDEL-sequence. In the ER where the pH is neutral, the proteins are not bound to the KDEL receptor, but under the slightly acidic conditions prevailing in the Golgi apparatus, the sequence will become bound to the receptor, which will then transport the proteins back to the ER where they are released. The KDEL receptor therefore has a function to retrieve ER luminal resident proteins that have escaped to the Golgi complex. Evidently, an external protein that contains this sequence might also

be transported to the ER if it has first reached the Golgi apparatus.

*Pseudomonas aeruginosa* exotoxin A contains a C-terminal sequence that resembles the KDEL sequence, namely REDLK. Experiments have shown that this sequence as such does not bind to the KDEL receptor, but if the C-terminal lysine is first removed by cellular exopeptidases, it binds, although not with high affinity. It was proposed that this sequence might direct the toxin to the ER (Chaudhary *et al*., 1990). In fact, removal of the REDLK sequence abolished the toxicity of the molecule, while replacing it with the sequence KDEL resulted in recovery of the toxicity. This strongly indicates that the transport to the ER is necessary for this toxin to act. It has, however, not been formally demonstrated that exotoxin A is really transported to the ER.

The first convincing evidence for transport of an external protein retrograde to the ER came with another toxin, Shiga toxin (Sandvig *et al*., 1992). This toxin has a structure that resembles that of cholera and heat-labile toxin, but it does not have a KDEL-related sequence. Also, this toxin binds to a glycolipid, and it had been known for some time that it is transported to the trans-Golgi region and possibly to the Golgi stacks. Treatment with butyric acid was found to sensitize some cells to the toxin, and upon analysis of butyric acid–treated cells that had been incubated with a conjugate of Shiga toxin and horseradish peroxidase, the conjugate was found in the ER, including the perinuclear space (Sandvig *et al*., 1992). Interestingly, the retrograde transport of Shiga toxin and cholera toxin was not inhibited by treatment with concanamycin that blocks the proton pump in intracellular vesicles, whereas the retrograde transport of furin and TGN38 was inhibited under these conditions (Schapiro *et al*., 1998).

Further evidence that toxins must pass the Golgi apparatus and the ER came from experiments with brefeldin A, which causes a disintegration of the Golgi apparatus. Treatment with this drug protected against intoxication of cells by ricin (Yoshida *et al*., 1991b). Furthermore, ricin containing signals for tyrosine sulfation and glycosylation modified accordingly after being added externally to the cells (Rapak *et al*., 1997).

## TRANSLOCATION TO THE CYTOSOL

### From the surface

So far only one bacterial toxin with an intracellular site of action has been demonstrated to be translocated from the cell surface, namely the invasive adenyl cyclase, CyaA, from *Bordetella pertussis*. This protein (177 kD) binds to, and apparently inserts into, membranes even at 4°C, as it cannot be removed by an alkaline carbonate wash. When the temperature is raised to 20°C or above, the toxin appears to translocate the enzymatically active part across the membrane. Calcium in the extracellular medium and an intact membrane potential are required for this process. Thus, when the cells were electrically depolarized, translocation did not occur. This raises the possibility that the enzymatically active part of the molecule is electrophorezed across the membrane, possibly through a pore. However, the electrical membrane potential affects the structure of all charged transmembrane molecules, and the role of the membrane potential could be an indirect one. It is interesting that translocation of fibroblast growth factors 1 and 2 also requires an intact membrane potential, in this case across the membrane of the endosomes (Malecki *et al*., 2002; Malecki *et al*., 2004). A 45 kD fragment of the *Bordetella pertussis* toxin carrying the enzymatic activity may subsequently be released into the cytosol (Rogel *et al*., 1992). Binding of calmodulin is required to activate the enzyme (Figure 7.3).

### From endosomes

Translocation from endosomes has been well characterized in two toxins, diphtheria toxin and anthrax toxin. In spite of their difference in primary structure and subunit composition, these two toxins are translocated by very similar mechanisms. Both toxins appear to be equipped with their own translocation devices. In the case of diphtheria toxin, this is part of the B-fragment, which is also involved in binding to receptors. The B-fragment contains a highly helical domain, the transmembrane or T-domain. At low pH as obtained in endosomes, this part of the toxin partially unfolds and inserts itself into the membrane forming an ion-conducting pore initiated by a hairpin of the hydrophobic helices 8 and 9 (Kaul *et al*., 1996). Hydrophobic photolabelling studies indicated that in addition to helices 8 and 9 that form the transmembrane hairpin, helices 1 and 5 also are inserted into the membrane, helix 1 in a transmembrane fashion (D'Silva and Lala, 1998; D'Silva and Lala, 2000). This brings the interfragment disulfide bond into the cytosol where it can be reduced to allow the A-fragment to be released into the cytosol (Figure 7.4). Hydrophobic and electrostatic interactions occur in a sequential manner in the process (Chenal *et al*., 2002; Ladokhin *et al*., 2004). The inserted T-domain may act as a membrane-inserted chaperone for the translocation of the A-fragment (Hammond *et al*., 2002; Rosconi *et al*., 2004). Although *in vitro* experiments with lipid films indicate that the toxin is able to

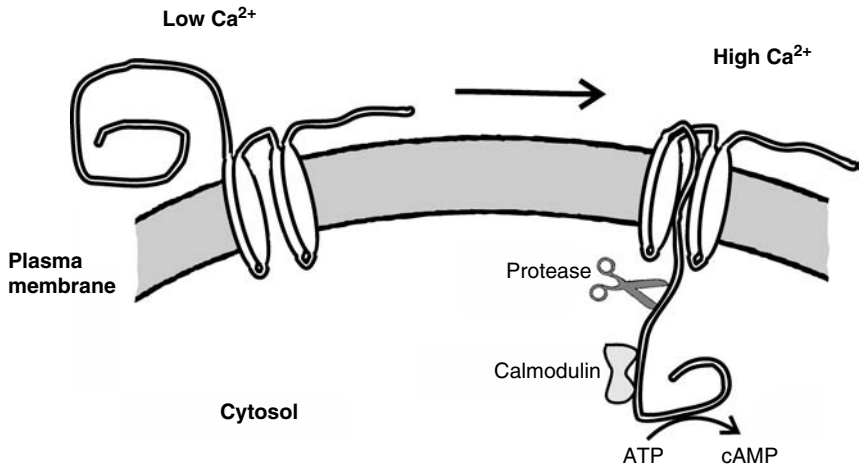



**FIGURE 7.3** Translocation of *Bordetella pertusssis* invasive adenyl cyclase.

translocate without assistance from other proteins (Senzel *et al*., 2000), in the *in vivo* situation additional proteins may increase the efficiency of translocation. Thus, recent data indicate that heat shock protein 90 and thioreductin reductase may be involved in the translocation process (Ratts *et al*., 2003).

Although cytotoxic necrotizing factor I from *E. coli* is endocytosed by a mechanism not involving clathrin, it appears to translocate to the cytosol in a similar way as diphtheria toxin (Contamin *et al*., 2000; Pei *et al*., 2001).

Anthrax toxin belongs to the group of binary toxins. One of the parts, the protective antigen (PA), binds to receptors at the cell surface and is then cleaved to release a 20 kD fragment (Petosa *et al*., 1997). The rest of the molecule forms heptamers at the level of the cell surface and at the same time generates a binding site for the other part of the toxin. This may be the edema factor or the lethal factor. Three molecules of either one can bind to each heptamer. The heptamer formed by the cleaved PA inserts into the membrane at low pH and forms ion-conducting channels in the membrane

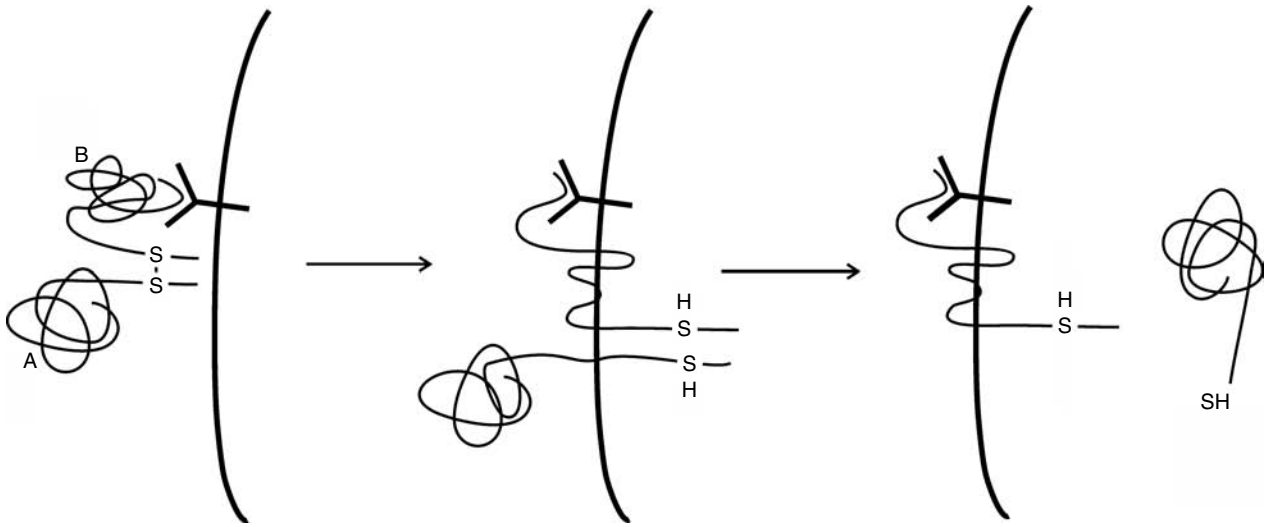



**FIGURE 7.4** Current model of translocation of diphtheria toxin. The toxin binds by its B-fragment to cell surface receptors and is endocytosed. Upon exposure to the low pH in endosomes, the helical T-domain is exposed and inserts into the membrane, pulling the C-terminal end of the A-fragment across the membrane. The disulfide bond is reduced upon exposure to the reducing conditions in the cytosol, and the remaining part of the A-fragment is translocated to the cytosol.

in a very similar way as diphtheria toxin. The enzymatically active part is translocated to the cytosol in the process (Figure 7.5). The heptameric PA appears to form a beta-barrel across the membrane, which could form the channel (Nassi *et al*., 2002). Certain mutations in PA that abolished the channel formation protected against toxicity, in spite of the fact that they did not impair binding of PA to the receptor (Sellman *et al*., 2001).

The C2 toxin from *Clostridium botulinum* is a binary toxin that resembles anthrax toxin but has another intracellular target: it ADP-ribosylates G-actin and induces depolymerization of actin. This toxin appears to enter by a mechanism similar to that of anthrax toxin (Blocker *et al*., 2003; Barth *et al*., 2000). Like anthrax toxin, it can carry passenger proteins into the cytosol (Haug *et al*., 2003b), and as in the case of diphtheria toxin, the chaperone Hsp90 is required for efficient translocation (Haug *et al*., 2003a).

In both the case of diphtheria toxin and anthrax toxin, it is necessary that the enzymatically active part is able to unfold during translocation. Thus, when disulfide-loops are introduced into the A-fragment of diphtheria toxin (it normally does not contain any), the translocation is blocked (Falnes *et al*., 1994). This is also the case if a ligand that confers tight folding is bound to a passenger protein that can otherwise be translocated along with the toxin (Wiedlocha *et al*., 1992). In the case of anthrax toxin, a fusion protein of diphtheria toxin A-fragment and the N-terminal part of anthrax toxin lethal factor ($LF_N$) can be translocated into the cytosol, but also here the translocation is blocked by the introduction of a disulfide bridge in the molecule (Arora and Leppla, 1994; Wesche *et al*., 1998).

The *Helicobacter pylori* toxin, VacA, requires low pH for activation, which is required for translocation to the cytosol (McClain *et al*., 2000). A chloride-permeable channel is formed in the process (McClain *et al*., 2003; Kim *et al*., 2004).

## From the ER

It now appears that an increasing number of toxins are found to be translocated from the ER. Although several toxins have been demonstrated to be transported retrograde to the ER (Lencer *et al*., 2003), it has been more difficult to demonstrate that the toxins are

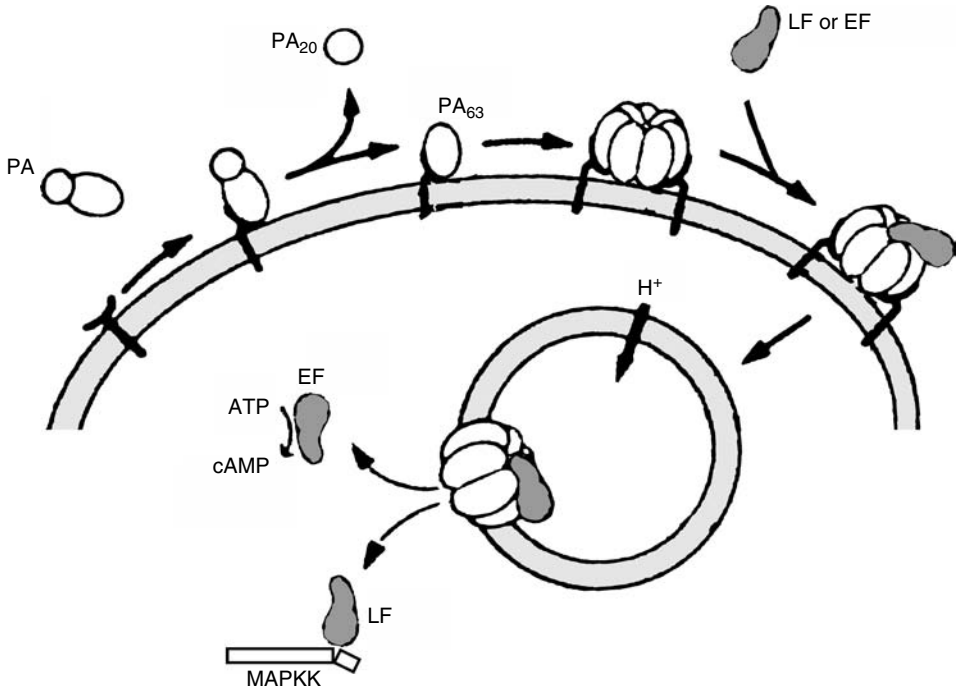



**FIGURE 7.5** Translocation of anthrax toxin. The protective antigen (PA) binds to cell surface receptors and a ~20 kD piece is cleaved off. The remaining part of the molecule aggregates with other cleaved PA molecules to form a heptamer, thereby generating binding sites for lethal factor (LF) and edema factor (EF). The complex is endocytosed. Upon acidification of the endosome, the heptamer inserts into the vesicle membrane and facilitates translocation of LF and EF to the cytosol (Petosa *et al*., 1997).

also translocated from this organelle. Certain plant toxins are very similar in structure and mechanism of action to bacterial toxins. One such plant toxin, ricin, acts on ribosomes in the same way as Shiga toxin, but it has a different B-subunit, which has lectin properties and binds to surface glycoproteins and glycolipids having terminal galactose residues (Olsnes *et al*., 1974). This toxin was the first to be shown to be transported retrograde from the cell surface to the trans-Golgi network (van Deurs *et al*., 1986; van Deurs *et al*., 1988). Some export proteins are sulfated on tyrosine and, since the sulfation occurs in the Golgi apparatus, a sulfation site was engineered into the C-terminal end of the toxin A-chain. By subsequently incubating the cells with labeled sulfate, those molecules that had been transported to the Golgi apparatus were selectively labeled. In other experiments where glycosylation sites were also engineered into the protein, it could be demonstrated that the labeled protein was partly glycosylated and that part of the A-chain molecules could be recovered from the cytosol (Rapak *et al*., 1997). Importantly, mainly the glycosylated form was found in the cytosol, indicating that only molecules that had been in the ER were translocated. It is therefore likely that the ER is the site of translocation. Calreticulin appears to be involved in the transport of ricin from the Golgi to the ER (Day *et al*., 2001).

This conclusion has become even more likely since it was found that misfolded export proteins are translocated retrograde from the ER to the cytosol where they become degraded by the proteasomes (Hiller *et al*., 1996; Knop *et al*., 1996; Werner *et al*., 1996). A membrane-associated ubiquitinating enzyme appears to be important for this process. It is conceivable that toxins such as ricin utilize this transport mechanism to penetrate the ER membrane. If that is so, the toxin must somehow avoid degradation by the proteasomes.

It was indeed found that ricin A-chain that had been glycosylated in the ER interacted with sec61p (Wesche *et al*., 1999). Evidence that this pathway is used for ricin and cholera toxin was also obtained (Simpson *et al*., 1999; Schmitz *et al*., 2000; Teter *et al*., 2002).

A yeast killer toxin, K28, also requires retrograde transport to the ER where it is translocated into the cytosol by a mechanism requiring Sec61 and luminal chaperones (Eisfeld *et al*., 2000). The internalized toxin irreversibly blocks DNA synthesis.

Very recently, evidence has appeared that retrograde translocation of misfolded proteins from the ER to the cytosol involves the transmembrane protein Derlin-1(Lilley and Ploegh, 2004; Ye *et al*., 2004). It will be interesting to see if this protein is also involved in the translocation of toxins.

## STABILITY OF TOXINS IN THE CYTOSOL

When wild type diphtheria toxin A-fragment was microinjected into cells, it proved to be exceptionally stable. The natural A-fragment has an N-terminal glycine residue that is stabilizing according to the N-end rule. However, even placing a destabilizing amino acid at the N-terminus of the A-fragment did not result in very rapid degradation. However, when a Flag-peptide was fused to the N-terminus, the N-terminal amino acid became very important for the stability (Falnes *et al*., 1998). When, in this case, a destabilizing amino acid was placed N-terminally, the toxic effect of the molecule was strongly reduced.

A survey of A-chains that are translocated to the cytosol revealed that there was not a strong tendency to avoid destabilizing amino acids at the N-terminal end. A possible reason for this is that a lysine residue that could serve as a target for ubiquitinylation is not present in a favorable distance from the N-terminus. When a lysine was inserted into ricin A-chain at a position favorable for ubiquitinylation, ricin was less toxic than the wild type toxin (Deeks *et al*., 2002). In fact, it appears that toxins that may be translocated from the ER and therefore exposed to the membrane-bound ubiquitinylating enzyme contain very few lysine residues, whereas diphtheria toxin, which is translocated from endosomes and therefore possibly less exposed to ubiquitinylation, contains a normal amount of lysines (Hazes and Read, 1997).

We found that ricin translocated to the cytosol accumulates to a larger extent in the presence of lactacystin that inhibits the proteasomes than in its absence (Wesche *et al*., 1999). Therefore, some degradation of the translocated ricin apparently takes place in the absence of the inhibitor. In accordance with this, the cells were also somewhat sensitized to ricin in the presence of lactacystin.

## TRANSLOCATION OF FUSION PROTEINS

It is an old concept that antibodies could be used to target toxins to cells of interest, such as cancer cells (Ehrlich, 1957; Olsnes, 2004). Originally, it was thought that it is sufficient that the antibody is efficient in targeting the molecule to the surface of the target cells. From our present knowledge, this is only part of the problem.

Immunotoxins may consist of an antibody linked to a whole toxin or only to its enzymatically active effector part. In the first case, the selectivity is usually low as the conjugate will bind to the toxin receptors in addition to

the target for the antibody. Higher selectivity is often obtained by linking only the effector moiety of the toxin to the antibody (Olsnes *et al*., 1989). However, in this case the conjugate does not have a translocation moiety, and it is dependent upon the translocation ability of the target cells. It is likely that in this case the conjugate must be transported to the ER for translocation to take place. This means that the antibody must be directed against a cell surface molecule that is able to direct the conjugate retrograde to the ER. So far no such protein has been described, whereas in the case of lipids such transport appears to take place. A number of natural toxins are, in fact, bound to cell surface lipids.

In the future design of immunotoxins, it will be advisable to look for molecules that are able to route the conjugate retrograde or to crosslink the receptor to another molecule that is able to direct the whole complex to the ER. A chimeric antibody reacting with both a cell-specific antigen and a molecule that is targeted to the ER could be able to carry out this task.

A more recent development in the field of toxin applications is to use toxins or their mutated, non-toxic counterparts to carry peptides into the cytosol for presentation by MHC class I molecules (Stenmark *et al*., 1991). There is a continuous breakdown of proteins in the cytosol, and some of the peptides produced by the proteasomes in this process are transported into the ER by the TAP transporters (Römisch, 1994) and become bound to newly synthesized MHC class 1 proteins. The complex, together with β2-microglobulin, is transported to the cell surface where it is presented to cytotoxic lymphocytes that recognize the complex if it contains a peptide not present in the normal complement of proteins. Foreign proteins that appear in the cytosol, such as viral proteins, and mutated autologous proteins, such as in cancer, are also broken down. Furthermore, when their peptides are presented at the cell surface, they are recognized as foreign, and the cell is destroyed before the virus can multiply or before the malignant cell can develop into a tumor. Exposure to a foreign peptide presented in this way leads to expansion of the relevant cytotoxic T-cell clone, i.e., an immunization effect.

It has been difficult to obtain a vaccination effect in the MHC class 1 system because the protein in question must be transported to the cytosol to be chopped up by the proteasomes into suitable pieces to bind to the MHC class 1 molecules. The property of protein toxins to enter the cytosol opened up the possibility using toxins as carrier molecules to transport passenger peptides and proteins into the cytosol (Stenmark *et al*., 1991; Wiedlocha *et al*., 1992; Klingenberg and Olsnes, 1996; de Haan and Hirst, 2002). The possibility of detoxifying the molecules without depriving them of their ability to be translocated could allow the development of a vaccine that does not have the potential side effects inherent in the use of attenuated viruses and injection of nucleic acids.

In attempts to test out the principle, various peptides that are known to be presented by MHC class 1 molecules were fused to the N-terminus of the A-fragment of diphtheria toxin. After binding the recombinant toxins to cells, it was possible to induce translocation of the fusion proteins into the cytosol in good yield (Stenmark *et al*., 1991). Attempts to demonstrate presentation by class 1 molecules were, however, unsuccessful.

Later, it was shown with anthrax toxin and with *Bordetella pertussis* invasive adenylate cyclase that peptides fused to the toxins are indeed able to induce a class 1 immune response in mice (Ballard *et al*., 1996; Ballard *et al*., 1998; Saron *et al*., 1997; Lu *et al*., 2000). This indicates, but does not prove; that the toxins transported the peptides to the cytosol by the toxin pathway. As mentioned previously, it now appears that proteins that are misfolded in the ER are translocated backwards into the cytosol where they are degraded by the proteasomes. Clearly, therefore, if a fusion protein is transported retrograde to the ER, it may be transported into the cytosol either as a conjugate with the toxin or after partial proteolytic degradation that could take place either at the level of endosomes in the Golgi apparatus or in the ER.

Whole proteins, such as acidic fibroblast growth factor (Wiedlocha *et al*., 1992), dihydrofolate reductase (Klingenberg *et al*., 1996), and a second A-fragment of diphtheria toxin (Madshus *et al*., 1992) can be translocated into cells by the toxin pathway. Unfortunately, most whole proteins that were fused to the toxin were not translocated (Wesche *et al*., 1998). Apparently, the reason for this is that the proteins must be able to unfold at low pH to follow the A-fragment across the membrane, and most proteins are not able to do this.

## ACKNOWLEDGMENTS

This work was supported by The Norwegian Cancer Society, The Research Council of Norway, Novo Nordisk Foundation, Blix Fund for the Promotion of Medical Research, Rachel and Otto Kr. Bruun's Fund, Torsteds Fund, and by The Jahre Foundation.

## REFERENCES

Akazawa, H., Komazaki, S., Shimomura, H., Terasaki, F., Zou, Y., Takano, H., Nagai, T. and Komuro, I. (2004). Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure. *J. Biol. Chem*. **279,** 41095–41103.

Aktories, K., Barmann, M., Ohishi, I., Tsuyama, S., Jakobs, K.H. and Habermann, E. (1986). Botulinum C2 toxin ADP-ribosylates actin. *Nature* **322,** 390–392.

Aktories, K., Mohr, C. and Koch, G. (1992). Clostridium botulinum C3 ADP-ribosyltransferase. *Curr. Top. Microbiol. Immunol*. **175,** 115–131.

Allured, V.S., Collier, R.J., Carroll, S.F. and McKay, D.B. (1986). Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution. *Proc. Natl. Acad. Sci. USA* **83,** 1320–1324.

Arora, N. and Leppla, S.H. (1994). Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun*. **62,** 4955–4961.

Ballard, J.D., Collier, R.J. and Starnbach, M.N. (1996). Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope *in vivo*. *Proc. Natl. Acad. Sci. USA* **93,** 12531–12534.

Ballard, J.D., Doling, A.M., Beauregard, K., Collier, R.J. and Starnbach, M.N. (1998). Anthrax toxin-mediated delivery *in vivo* and *in vitro* of a cytotoxic T-lymphocyte epitope from ovalbumin. *Infection and Immunity* **66,** 615–619.

Bann, J.G. and Hultgren, S.J. (2004). Structural biology: anthrax hijacks host receptor. *Nature* **430,** 843–844.

Barth, H., Blocker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R. and Aktories, K. (2000). Cellular uptake of Clostridium botulinum C2 toxin requires oligomerization and acidification. *J. Biol. Chem*. **275,** 18704–18711.

Blocker, D., Pohlmann, K., Haug, G., Bachmeyer, C., Benz, R., Aktories, K. and Barth, H. (2003). Clostridium botulinum C2 toxin: low pH-induced pore formation is required for translocation of the enzyme component C2I into the cytosol of host cells. *J. Biol. Chem*. **278,** 37360–37367.

Boquet, P., Ricci, V., Galmiche, A. and Gauthier, N.C. (2003). Gastric cell apoptosis and H-pylori: has the main function of VacA finally been identified? *Trends Microbiol*. **11,** 410–413.

Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, J.A. (2001). Identification of the cellular receptor for anthrax toxin. *Nature* **414,** 225–229.

Cha, J.H., Chang, M.Y., Richardson, J.A. and Eidels, L. (2003). Transgenic mice expressing the diphtheria toxin receptor are sensitive to the toxin. *Mol. Microbiol*. **49,** 235–240.

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989). The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J*. **8,** 1087–1092.

Chaudhary, V.K., Jinno, Y., FitzGerald, D. and Pastan, I. (1990). Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA* **87,** 308–312.

Chenal, A., Savarin, P., Nizard, P., Guillain, F., Gillet, D. and Forge, V. (2002). Membrane protein insertion regulated by bringing electrostatic and hydrophobic interactions into play. A case study with the translocation domain of diphtheria toxin. *J. Biol. Chem*. **277,** 43425–43432.

Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M., Kantardjieff, K. A., Collier, R.J. and Eisenberg, D. (1992). The crystal structure of diphtheria toxin. *Nature* **357,** 216–222.

Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. *Bacteriol. Rev*. **39,** 54–85.

Contamin, S., Galmiche, A., Doye, A., Flatau, G., Benmerah, A. and Boquet, P. (2000). The p21 Rho-activating toxin cytotoxic necrotizing factor 1 is endocytosed by a clathrin-independent mechanism and enters the cytosol by an acidic-dependent membrane translocation step. *Mol. Biol. Cell* **11,** 1775–1787.

D'Silva, P.R. and Lala, A.K. (1998). Unfolding of diphtheria toxin. Identification of hydrophobic sites exposed on lowering of pH by photolabeling. *J. Biol. Chem*. **273,** 16216–16222.

D'Silva, P.R. and Lala, A.K. (2000). Organization of diphtheria toxin in membranes. A hydrophobic photolabeling study. *J. Biol. Chem*. **275,** 11771–11777.

Day, P.J., Owens, S.R., Wesche, J., Olsnes, S., Roberts, L. M. and Lord, J. M. (2001). An interaction between ricin and calreticulin that may have implications for toxin trafficking. *J. Biol. Chem*. **276,** 7202 -7208.

de Bernard, M., Moschioni, M., Napolitani, G., Rappuoli, R. and Montecucco, C. (2000). The VacA toxin of Helicobacter pylori identifies a new intermediate filament-interacting protein. *EMBO J*. **19,** 48–56.

de Haan, L. and Hirst, T.R. (2002). Bacterial toxins as versatile delivery vehicles. *Curr. Opin. Drug Discov. Devel*. **5,** 269– 278.

Deeks, E.D., Cook, J.P., Day, P.J., Smith, D.C., Roberts, L.M. and Lord, J.M. (2002). The low lysine content of ricin A chain reduces the risk of proteolytic degradation after translocation from the endoplasmic reticulum to the cytosol. *Biochemistry* **41,** 3405–3413.

DeGrandis, S., Law, H., Brunton, J., Gyles, C. and Lingwood, C.A. (1989). Globotetraosylceramide is recognized by the pig edema disease toxin. *J. Biol. Chem*. **264,** 12520–12525.

Draper, R.K. and Simon, M.I. (1980). The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. *J. Cell. Biol*. **87,** 849–854.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D. and Vande Woude, G.F. (1998). Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280,** 734–737.

Ehrlich, P. (1957). Himmelweit, F. (ed.): The collected papers of Paul Ehrlich. Pergamon Press, London.

Eisfeld, K., Riffer, F., Mentges, J. and Schmitt, M. J. (2000). Endocytotic uptake and retrograde transport of a virally encoded killer toxin in yeast. *Mol. Microbiol*. **37,** 926–940.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988). Site of action of a Vero toxin (VT2) from Escherichia coli O157: H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. *Eur. J. Biochem*. **171,** 45–50.

Falnes, P.O., Madshus, I.H., Wilson, B.A. and Olsnes, S. (1994). Inhibition of membrane translocation of diphtheria toxin A-fragment by internal disulfide bridges. *J. Biol. Chem*. **269,** 8402–8407.

Falnes, P.O., and Olsnes, S. (1998). Modulation of the intracellular stability and toxicity of diphtheria toxin through degradation by the N-end rule pathway. *EMBO J*. **17,** 615–625.

Fiorentini, C., Fabbri, A., Matarrese, P., Falzano, L., Boquet, P. and Malorni, W. (1997). Hindrance of apoptosis and phagocytic behavior induced by Escherichia coli cytotoxic necrotizing factor 1: Two related activities in epithelial cells. *Biochem. Biophys. Res. Commun*. **241,** 341–346.

Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Florentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387,** 729–733.

Hammond, K., Caputo, G.A. and London, E. (2002). Interaction of the membrane-inserted diphtheria toxin T domain with peptides and its possible implications for chaperone-like T domain behavior. *Biochemistry* **41,** 3243–3253.

Haug, G., Leemhuis, J., Tiemann, D., Meyer, D.K., Aktories, K. and Barth, H. (2003a). The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol. *J. Biol. Chem*. **278,** 32266–32274.

Haug, G., Wilde, C., Leemhuis, J., Meyer, D.K., Aktories, K. and Barth, H. (2003b). Cellular uptake of Clostridium botulinum C2 toxin: membrane translocation of a fusion toxin requires

unfolding of its dihydrofolate reductase domain. *Biochemistry* **42,** 15284–15291.

Hazes, B. and Read, R.J. (1997). Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry* **36,** 11051–11054.

Hiller, M., Finger, A., Schweiger, M. and Wolf, D. (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science* **273,** 1725–1728.

Hooper, K.P. and Eidels, L. (1996). Glutamic acid 141 of the diphtheria toxin receptor (HB-EGF precursor) is critical for toxin binding and toxin sensitivity. *Biochemical and Biophysical Research Communications* **220,** 675–680.

Hughes, R.C. and Gardas, A. (1976). Phenotypic reversion of ricin-resistant hamster fibroblasts to a sensitive state after coating with glycolipid receptors. *Nature* **264,** 63–66.

Inoue, S., Sugai, M., Murooka, Y., Paik, S.Y., Hong, Y.M., Ohgai, H. and Suginaka, H. (1991). Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from Staphylococcus aureus. *Biochem. Biophys. Res. Commun*. **174,** 459–464.

Joseph, K.C., Stieber, A. and Gonatas, N.K. (1979). Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with GM1 ganglioside. Cholera toxin internalization into Neuroblastoma GERL. *J. Cell. Biol*. **81,** 543–554.

Just, I., Mohr, C., Schallehn, G., Menard, L., Didsbury, J.R., Vandekerckhove, J., van Damme, J. and Aktories, K. (1992a). Purification and characterization of an ADP-ribosyltransferase produced by Clostridium limosum. *J. Biol. Chem*. **267,** 10274–10280.

Just, I., Schallehn, G. and Aktories, K. (1992b). ADP-ribosylation of small GTP-binding proteins by Bacillus cereus. *Biochem. Biophys. Res. Commun*. **183,** 931–936.

Just, I., Selzer, J., Hofmann, F., Green, G.A. and Aktories, K. (1996). Inactivation of Ras by Clostridium sordellii lethal toxin-catalyzed glucosylation. *J. Biol. Chem*. **271,** 10149–10153.

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995a). Glucosylation of Rho proteins by Clostridium difficile toxin B. *Nature* **375,** 500–503.

Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995b). The enterotoxin from Clostridium difficile (ToxA) monoglucosylates the Rho proteins. *J. Biol. Chem*. **270,** 13932–13936.

Kaul, P., Silverman, J., Shen, W.H., Blanke, S.R., Huynh, P.D., Finkelstein, A. and Collier, R.J. (1996). Roles of Glu 349 and Asp 352 in membrane insertion and translocation by diphtheria toxin. *Protein Sci*. **5,** 687–692.

Kim, K. and Groman, N.B. (1965). *In vitro* inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol*. **90,** 1552–1556.

Kim, S., Chamberlain, A.K. and Bowie, J.U. (2004). Membrane channel structure of Helicobacter pylori vacuolating toxin: role of multiple GXXXG motifs in cylindrical channels. *Proc. Natl. Acad. Sci. USA* **101,** 5988–5991.

Klingenberg, O. and Olsnes, S. (1996). Ability of methotrexate to inhibit translocation to the cytosol of dihydrofolate reductase fused to diphtheria toxin. *Biochem. J*. **313,** 647–653.

Knop, M., Finger, A., Braun, T., Hellmuth, K. and Wolf, D.H. (1996). Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J*. **15,** 753–763.

Koehler, T.M. and Collier, R.J. (1991). Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. *Mol. Microbiol*. **5,** 1501–1506.

Kounnas, M.Z., Morris, R.E., Thompson, M.R., FitzGerald, D.J., Strickland, D.K. and Saelinger, C.B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem*. **267,** 12420–12423.

Lacy, D.B., Wigelsworth, D.J., Scobie, H.M., Young, J.A. and Collier, R.J. (2004). Crystal structure of the von Willebrand factor A domain of human capillary morphogenesis protein 2: an anthrax toxin receptor. *Proc. Natl. Acad. Sci. USA* **101,** 6367–6372.

Ladokhin, A.S., Legmann, R., Collier, R.J. and White, S.H. (2004). Reversible refolding of the diphtheria toxin T-domain on lipid membranes. *Biochemistry* **43,** 7451–7458.

Lanzrein, M., Sand, O. and Olsnes, S. (1996). GPI-anchored diphtheria toxin receptor allows membrane translocation of the toxin without detectable ion channel activity. *EMBO J*. **15,** 725–734.

Lemichez, E., Flatau, G., Bruzzone, M., Boquet, P. and Gauthier, M. (1997). Molecular localization of the Escherichia coli cytotoxic necrotizing factor CNF1 cell-binding and catalytic domains. *Mol. Microbiol*. **24,** 1061–1070.

Lencer, W.I. and Tsai, B. (2003). The intracellular voyage of cholera toxin: going retro. *Trends Biochem. Sci*. **28,** 639–645.

Leppla, S.H. (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **79,** 3162–3166.

Li, J. D., Carroll, J. and Ellar, D. J. (1991). Crystal structure of insecticidal delta-endotoxin from Bacillus thuringiensis at 2.5 A resolution. *Nature* **353,** 815–821.

Lilley, B.N. and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429,** 834–840.

Ling, H., Boodhoo, A., Hazes, B., Cummings, M.D., Armstrong, G.D., Brunton, J.L. and Read, R.J. (1998). Structure of the Shiga-like toxin-I B-pentamer complexed with an analog of its receptor GB3. *Biochemistry* **37,** 1777–1788.

Lu, Y., Friedman, R., Kushner, N., Doling, A., Thomas, L., Touzjian, N., Starnbach, M. and Lieberman, J. (2000). Genetically modified anthrax lethal toxin safely delivers whole HIV protein antigens into the cytosol to induce T cell immunity. *Proc. Natl. Acad. Sci. USA* **97,** 8027–8032.

Madshus, I.H., Olsnes, S. and Stenmark, H. (1992). Membrane translocation of diphtheria toxin carrying passenger protein domains. *Infect. Immun*. **60,** 3296–3302.

Malecki, J., Wesche, J., Skjerpen, C.S., Wiedlocha, A. and Olsnes, S. Translocation of FGF-1 anf FGF-2 across vesicular membranes occurs during G1-phase by a common mechanism. *Molecular Biology of the Cell*. 2004.**15,** 801–814.

Malecki, J., Wiedlocha, A., Wesche, J. and Olsnes, S. (2004). Vesicle transmembrane potential is required for translocation to cytosol of externally added FGF-1. *EMBO Journal*, **15,** 801–814.

McClain, M. S., Iwamoto, H., Cao, P., Vinion-Dubiel, A.D., Li, Y., Szabo, G., Shao, Z. and Cover, T. L. (2003). Essential role of a GXXXG motif for membrane channel formation by Helicobacter pylori vacuolating toxin. *J. Biol. Chem*. **278,** 12101–12108.

McClain, M.S., Schraw, W., Ricci, V., Boquet, P. and Cover, T.L. (2000). Acid activation of Helicobacter pylori vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells. *Mol. Microbiol*. **37,** 433–442.

Meager, A., Ungkitchanukit, A. and Hughes, R.C. (1976). Variants of hamster fibroblasts resistant to Ricinus communis toxin (ricin). *Biochem. J*. **154,** 113–124.

Merritt, E.A. and Hol, W. G. (1995). $AB_5$ toxins. *Current Biology* **5,** 165–171.

Milne, J.C. and Collier, R.J. (1993). pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. *Mol. Microbiol*. **10,** 647–653.

Montecucco, C. and Schiavo, G. (1993). Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem. Sci*. **18,** 324–327.

Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. and Boquet, P. (1985). Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell. Biol*. **101,** 548–559.

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding, EGF-like growth factor precursor. *Cell* **69,** 1051–1061.

Nambiar, M.P. and Wu, H.C. (1995). Ilimaquinone inhibits the cytotoxicities of ricin, diphtheria toxin, and other protein toxins in Vero cells. *Exp. Cell Res*. **219,** 671–678.

Nassi, S., Collier, R.J. and Finkelstein, A. (2002). PA63 channel of anthrax toxin: an extended beta-barrel. *Biochemistry* **41,** 1445–1450.

Nishiki, T., Kamata, Y., Nemoto, Y., Omori, A., Ito, T., Takahashi, M. and Kozaki, S. (1994). Identification of protein receptor for Clostridium botulinum type B neurotoxin in rat brain synaptosomes. *J. Biol. Chem*. **269,** 10498–10503.

Olsnes, S. (2004). The history of ricin, abrin, and related toxins. *Toxicon* **44,** 361–370.

Olsnes, S. and Refsnes, K. (1978). On the mechanism of toxin resistance in cell variants resistant to abrin and ricin. *Eur. J. Biochem*. **88,** 7–15.

Olsnes, S., Refsnes, K. and Pihl, A. (1974). Mechanism of action of the toxic lectins abrin and ricin. *Nature* **249,** 627–631.

Olsnes, S., Reisbig, R. and Eiklid, K. (1981). Subunit structure of Shigella cytotoxin. *J. Biol. Chem*. **256,** 8732–8738.

Olsnes, S., Sandvig, K., Petersen, O.W. and van Deurs, B. (1989). Immunotoxins—entry into cells and mechanisms of action. *Immunol. Today* **10,** 291–295.

Papini, E., Zoratti, M. and Cover, T.L. (2001). In search of the Helicobacter pylori VacA mechanism of action. *Toxicon* **39,** 1757–1767.

Pappenheimer, A. M., Jr. (1977). Diphtheria toxin. *Annu. Rev. Biochem*. **46,** 69–94.

Pei, S., Doye, A. and Boquet, P. (2001). Mutation of specific acidic residues of the CNF1 T domain into lysine alters cell membrane translocation of the toxin. *Mol. Microbiol*. **41,** 1237–1247.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Popoff, M.R., Chaves-Olarte, E., Lemichez, E., von Eichel-Streiber, C., Thelestam, M., Chardin, P., Cussac, D., Antonny, B., Chavrier, P., Flatau, G., Giry, M., de Gunzburg, J. and Boquet, P. (1996). Ras, Rap, and Rac small GTP-binding proteins are targets for Clostridium sordellii lethal toxin glucosylation. *J. Biol. Chem*. **271,** 10217–10224.

Raab, G. and Klagsbrun, M. (1997). Heparin-binding, EGF-like growth factor. *Biochim. Biophys. Acta*. **1333**:F179–99.

Rapak, A., Falnes, P.O. and Olsnes, S. (1997). Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc. Natl. Acad. Sci. USA* **94,** 3783–3788.

Ratts, R., Zeng, H., Berg, E.A., Blue, C., McComb, M.E., Costello, C.E., vanderSpek, J.C. and Murphy, J.R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J. Cell. Biol*. **160,** 1139–1150.

Reisbig, R., Olsnes, S. and Eiklid, K. (1981). The cytotoxic activity of Shigella toxin. Evidence for catalytic inactivation of the 60 S ribosomal subunit. *J. Biol. Chem*. **256,** 8739–8744.

Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E. and Boquet, P. (2000). High cell sensitivity to Helicobacter pylori VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol. Biol. Cell*. **11,** 3897–3909.

Rogel, A. and Hanski, E. (1992). Distinct steps in the penetration of adenylate cyclase toxin of Bordetella pertussis into sheep erythrocytes. Translocation of the toxin across the membrane. *J. Biol. Chem*. **267,** 22599–22605.

Rogel, A., Meller, R. and Hanski, E. (1991). Adenylate cyclase toxin from Bordetella pertussis. The relationship between induction of cAMP and hemolysis. *J. Biol. Chem*. **266,** 3154–3161.

Römisch, K. (1994). Peptide traffic across the ER membrane:TAPs and other conduits. *Trends Cell Biol*. **4,** 311–314.

Rosconi, M.P., Zhao, G. and London, E. (2004). Analyzing topography of membrane-inserted diphtheria toxin T domain using BODIPY-streptavidin: at low pH, helices 8 and 9 form a transmembrane hairpin but helices 5–7 form stable nonclassical inserted segments on the cis side of the bilayer. *Biochemistry* **43,** 9127–9139.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A. and Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol*. **19,** 746–750.

Sandvig, K., Garred, O., Prydz, K., Kozlov, J. V., Hansen, S. H. and van Deurs, B. (1992). Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* **358,** 510–512.

Sandvig, K. and Olsnes, S. (1979). Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. *Exp. Cell. Res*. **121,** 15–25.

Sandvig, K. and Olsnes, S. (1980). Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell. Biol*. **87,** 828–832.

Sandvig, K. and Olsnes, S. (1982a). Entry of the toxic proteins abrin, modeccin, ricin, and diphtheria toxin into cells. I. Requirement for calcium. *J. Biol. Chem*. **257,** 7495–7503.

Sandvig, K. and Olsnes, S. (1982b). Entry of the toxic proteins abrin, modeccin, ricin, and diphtheria toxin into cells. II. Effect of pH, metabolic inhibitors, and ionophores and evidence for toxin penetration from endocytotic vesicles. *J. Biol. Chem*. **257**: 7504–7513.

Sandvig, K., Olsnes, S., Petersen, O. W. and van Deurs, B. (1989). Control of coated-pit function by cytoplasmic pH. *Methods Cell. Biol*. **32,** 365–382.

Sandvig, K., Sundan, A. and Olsnes, S. (1984). Evidence that diphtheria toxin and modeccin enter the cytosol from different vesicular compartments. *J. Cell. Biol*. **98,** 963–970.

Santelli, E., Bankston, L.A., Leppla, S.H. and Liddington, R.C. (2004). Crystal structure of a complex between anthrax toxin and its host cell receptor. *Nature* **430,** 905–908.

Saron, M.F., Fayolle, C., Sebo, P., Ladant, D., Ullmann, A. and Leclerc, C. (1997). Anti-viral protection conferred by recombinant adenylate cyclase toxins from Bordetella pertussis carrying a CD8+ T cell epitope from lymphocytic choriomeningitis virus. *Proc. Natl. Acad. Sci. USA* **94,** 3314–3319.

Schapiro, F.B., Lingwood, C., Furuya, W. and Grinstein, S. (1998). pH-independent retrograde targeting of glycolipids to the Golgi complex. *Am. J. Physiol*. **274**:C319–32.

Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L. and Montecucco, C. (1992). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. *EMBO J*. **11,** 3577–3583.

Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M. and Aktories, K. (1997). Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1. *Nature* **387,** 725–729.

Schmitz, A., Herrgen, H., Winkeler, A. and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell. Biol*. **148,** 1203–1212.

Scobie, H.M., Rainey, G.J., Bradley, K.A. and Young, J.A. (2003). Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc. Natl. Acad. Sci. USA* **100**: 5170–5174.

Sellman, B.R., Nassi, S. and Collier, R.J. (2001). Point mutations in anthrax protective antigen that block translocation. *J. Biol. Chem*. **276,** 8371–8376.

Selzer, J., Hofmann, F., Rex, G., Wilm, M., Mann, M., Just, I. and Aktories, K. (1996). Clostridium novyi alpha-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. *J. Biol. Chem*. **271,** 25173–25177.

Senzel, L., Gordon, M., Blaustein, R.O., Oh, K.J., Collier, R.J. and Finkelstein, A. (2000). Topography of diphtheria toxin's T domain in the open channel state. *J. Gen. Physiol*. **115,** 421–434.

Simpson, J.C., Roberts, L.M., Romisch, K., Davey, J., Wolf, D.H. and Lord, J.M. (1999) Ricin A chain utilizes the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett*. **459,** 80–84.

Stenmark, H., Moskaug, J.O., Madshus, I.H., Sandvig, K. and Olsnes, S. (1991). Peptides fused to the amino-terminal end of diphtheria toxin are translocated to the cytosol. *J. Cell. Biol*. **113,** 1025–1032.

Takahashi, T., Umata, T. and Mekada, E. (2001). Extension of juxtamembrane domain of diphtheria toxin receptor arrests translocation of diphtheria toxin fragment A into cytosol. *Biochem. Biophys. Res. Commun*. **281,** 690–696.

Takemura, T., Kondo, S., Homma, T., Sakai, M. and Harris, R. C. (1997). The membrane-bound form of heparin-binding epidermal growth factor-like growth factor promotes survival of cultured renal epithelial cells. *J. Biol. Chem*. **272,** 31036–31042.

Teter, K., Allyn, R.L., Jobling, M.G. and Holmes, R.K. (2002). Transfer of the cholera toxin A1 polypeptide from the endoplasmic reticulum to the cytosol is a rapid process facilitated by the endoplasmic reticulum-associated degradation pathway. *Infection and Immunity*. **70,** 6166–6171.

van Deurs, B., Petersen, O.W., Olsnes, S. and Sandvig, K. (1989). The ways of endocytosis. *Int. Rev. Cytol*. **117,** 131–177.

van Deurs, B., Sandvig, K., Petersen, O.W., Olsnes, S., Simons, K. and Griffiths, G. (1988). Estimation of the amount of internalized ricin that reaches the trans-Golgi network. *J. Cell. Biol*. **106,** 253–267.

van Deurs, B., Tönnessen, T.I., Petersen, O.W., Sandvig, K. and Olsnes, S. (1986). Routing of internalized ricin and ricin conjugates to the Golgi complex. *J. Cell. Biol*. **102,** 37–47.

van Heyningen, W.E. (1974). Gangliosides as membrane receptors for tetanus toxin, cholera toxin, and serotonin. *Nature* **249,** 415–417.

Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980a). ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. *J. Biol. Chem*. **255,** 10717–10720.

Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980b). ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. *J. Biol. Chem*. **255,** 10710–10716.

Vitale, G., Pellizzari, R., Recchi, C., Napolitani, G., Mock, M. and Montecucco, C. (1998). Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun*. **248,** 706–711.

Werner, E.D., Brodsky, J.L. and McCracken, A.A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: An unconventional route to a familiar fate. *Proc. Natl. Acad. Sci. USA* **93,** 13797–13801.

Wesche, J., Elliot, J.L., Falnes, P.O., Olsnes, S. and Collier, R.J. (1998). Characterization of membrane translocation by anthrax protective antigen. *Biochemistry* **45,** 15737–15746.

Wesche, J., Rapak, A. and Olsnes, S. (1999). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J. Biol. Chem*. **274,** 34443–34449.

Wick, M. J., Hamood, A. N. and Iglewski, B. H. (1990). Analysis of the structure-function relationship of Pseudomonas aeruginosa exotoxin A. *Mol. Microbiol*. **4,** 527–535.

Wiedlocha, A., Madshus, I.H., Mach, H., Middaugh, C.R. and Olsnes, S. (1992). Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. *EMBO J*. **11,** 4835–4842.

Ye, Y.H., Shibata, Y., Yun, C., Ron, D. and Rapoport, T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841–847.

Yoshida, T., Chen, C., Zhang, M. and Wu, H.C. (1991a). Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and Pseudomonas toxin. *Exp. Cell. Res*. **192,** 389–395.

Yoshida, T., Chen, C.C., Zhang, M.S. and Wu, H.C. (1991b). Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and Pseudomonas toxin. *Exp. Cell. Res*. **192,** 389–395.

Youle, R.J. and Colombatti, M. (1987). Hybridoma cells containing intracellular anti-ricin antibodies show ricin meets secretory antibody before entering the cytosol. *J. Biol. Chem*. **262,** 4676–4682.

CHAPTER

# 8

# Intracellular trafficking of bacterial and plant protein toxins

*Christophe Lamaze and Ludger Johannes*

## INTRODUCTION

Intracellular trafficking is a very general but tightly regulated process used by a variety of molecules to cross the membranes of living cells. This process is therefore crucial to a large group of bacterial and plant toxins that need to be translocated to the cytosol where their intracellular targets are located. As most toxins use pre-existing transport pathways, the study of these pathways has contributed to the identification and understanding of several novel transport mechanisms in mammalian cells, including endocytosis and intracellular distribution pathways. Over the last 10 years, tremendous progress has been made in the characterization of multiple endocytic processes together with the intracellular distribution of endocytosed molecules.

In this chapter, we will address the molecular mechanisms used by selected toxins for their endocytosis and intracellular distribution in mammalian cells. Whether these toxins inhibit protein synthesis (diphtheria toxin (DTx), Shiga toxin and Shiga-like toxin (STx and SLTx), ricin, and *Pseudomonas aeruginosa* exotoxin A (PEx)) or cell signaling events (cholera toxin (CTx), the *Escherichia coli* heat-labile toxin (LTx), pertussis toxin (CyaA), and anthrax toxin), they all rely on endocytosis and intracellular transport to gain access to the cytosol where they exert their poisonous action.

## GENERAL CONSIDERATIONS

The toxins described here are also called *A-B toxins*, as they are composed of a catalytically active A-subunit covalently or non-covalently bound to the B-subunit. Only the B-subunit is necessary for the binding, uptake, and cellular trafficking of the holotoxin. Very low concentrations of these toxins are required to induce toxicity, and very few internalized molecules are sufficient to kill the cell. For instance, one ricin molecule can inactivate 2,000 ribosomes in one minute and one molecule of the DTx catalytic fragment A can kill a cell in two days (Yamaizumi *et al*., 1978). This characteristic must be considered when studying the endocytosis of toxins, as most inhibitors used to block known endocytic routes still allow the entry of a few molecules. This is particularly a problem for toxins that cannot be labeled with probes used to trace internalized cargos and that can only be detected via their cytotoxic effect.

## CELL SURFACE RECEPTORS

The first membrane step in endocytosis is the binding of exogenous molecules to a cell surface receptor. The identification of toxin receptors provides important information as many characteristics of toxin transport are determined by the nature and the specific localization of the receptor. For instance, ricin, produced by the plant *Ricinus communis*, binds to galactose residues present in many glycoproteins and glycolipids, and as a result can interact with multiple sites of the plasma membrane. Hence, ricin is internalized by every operating endocytic pathway available and serves as a marker of overall cell endocytic activity. Most toxins bind to more restricted receptor structures. The

receptors of some toxins have not yet been identified. This is true for cytotoxic necrotizing factor 1 (CNF1), a type III single-chain toxin molecule (Falbo *et al*., 1993) that is produced by uropathogenic *E. coli* strains (Cid *et al*., 1996). CNF1 induces ruffling of eukaryotic cell membranes, actin stress fiber formation, and spreading and multinucleation through the permanent activation of the small GTPase Rho. CNF1 binds tightly to a yet unidentified cell surface receptor. The first 190 amino-terminal residues of the single chain toxin are necessary for its binding to the cell surface (Fabbri *et al*., 1999).

Some toxins, such as STx, CTx, and closely related LTx, bind to lipids, whereas others bind to transmembrane protein receptors. In the latter group, *Pseudomonas* exotoxin A binds to the low-density lipoprotein receptor-related protein (LRP) via its domain IV (Kounnas *et al*., 1992). Like the LRP receptor, PEx is probably also endocytosed by the clathrin-coated pit pathway (Conner and Schmid, 2003). *Bordetella pertussis*, the agent responsible for whooping cough, secretes an adenylate cyclase toxin (CyaA) that is one of the major virulence factors of this organism. After cell invasion, CyaA activates endogenous calmodulin and increases cAMP production in a non-regulated manner. It was recently demonstrated that CyaA uses the alpha (M)beta(2) integrin (CD11b/CD18) as a cell receptor and that the increase in intracellular cAMP level following CyaA intoxication can be specifically inhibited by anti-CD11b monoclonal antibodies (Guermonprez *et al*., 2001). It was subsequently shown that the acylation of CyaA is required for the interaction of the toxin with cells expressing CD11b and that the main integrin interacting domain of CyaA is located in its glycine/aspartate-rich repeat region (El-Azami-El-Idrissi *et al*., 2003). The uptake mechanism used by CyaA is unknown, but it is likely that the integrin receptor and its bound toxin are also endocytosed through clathrin-coated pits, as is the case for most integrins. The entry pathway used by CyaA may not be important for its translocation, as CyaA reaches the cytosol directly from the plasma membrane (Rogel and Hanski, 1992).

Some toxins, such as the anthrax toxin, have developed more sophisticated ways of entering cells. The anthrax toxin is one of two virulence factors produced by *Bacillus anthracis* (Leppla, 1988). This toxin consists of three subunits: edema factor (EF), the lethal factor (LF), and the protective antigen (PA). These three subunits interact in a complex manner that conditions toxin entry and toxicity (Kurzchalia, 2003). PA is an 83 kD protein (PA83) that is required for the recognition and the cellular transport of subunits EF and LF. PA binds to a widely expressed transmembrane receptor of 368 amino acids termed ATR for Anthrax Toxin Receptor (Bradley *et al*., 2001). After binding to ATR, PA is cleaved at the cell surface by furin proteases, resulting in a 63 kD form of PA that remains bound to ATR. This cleavage is crucial as it confers two new properties that determine subsequent toxicity. First, PA63, unlike PA83, can oligomerize to form heptamers, a state required for their association with EF and LF at the cell surface (Cunningham *et al*., 2002; Mogridge *et al*., 2001; Singh *et al*., 1994). Second, PA63 is endocytosed together with EF and LF, whereas PA83 remains at the cell surface (Milne *et al*., 1994).

The binding of some toxins to the cell surface requires the expression of specific lipids. The best-characterized lipid-binding toxins are Shiga toxin (STx), produced by *Shigella dysenteriae*, and cholera toxins (CTx), produced by *Vibrio cholerae*, which respectively bind to cells expressing Gb3 and GM1. The heat-labile toxin (LTx), which is closely related to CTx and produced by some strains of *Escherichia coli*, also uses the glycolipid GM1 as a receptor. Type II LTx toxins also bind to the GD1a and GD1b glycosphingolipids. The binding of these pentameric toxins can result in the clustering of the glycolipids, and it has been shown that CTx binding can lead to the relocalization of GM1 to raft-type membrane microdomains (see below) (Harder *et al*., 1998; Spiegel *et al*., 1984). STx, which also has a pentameric B-subunit and binds to the glycolipid receptor Gb3, is also constitutively localized to this type of microdomain (Falguières *et al*., 2001; Katagiri *et al*., 1999; Kovbasnjuk *et al*., 2001). Interestingly, the proportion of Gb3-bound STx associated with lipid rafts differs from cell to cell and may be an important determinant for the intracellular targeting of the toxin (Falguières *et al*., 2001) (see below). As GM1 is found in both caveolae and lipid rafts, it was assumed that CTx was internalized by these structures. However, several groups have shown that CTx and STx can also be endocytosed by clathrin-coated pits (see below). How CTx and STx are recruited into clathrin-coated pits and the role of lipid clustering in this recruitment need to be determined to understand the trafficking of these toxins. Recent data have confirmed that STx can be efficiently endocytosed in cells devoid of functional clathrin-coated pits (Saint-Pol *et al*., 2004). The role and the importance of this alternative endocytic pathway in cell intoxication by STx have yet to be determined.

## MULTIPLE ENDOCYTIC PATHWAYS OPERATE IN MAMMALIAN CELLS

It was long assumed that endocytosis involving the formation of clathrin-coated pits and vesicles was the only

efficient process for the internalization of exogenous molecules bound to their specific transmembrane receptors. Several recent reviews have described the enormous progress that has been made in the last 10 years in the molecular understanding of the clathrin-dependent pathway (Brodsky *et al*., 2001; Owen *et al*., 2004; Rappoport *et al*., 2004). Nevertheless, it has been known for almost 20 years that some toxins can enter cells by means other than the well-described clathrin-dependent endocytosis (Montesano *et al*., 1982; Moya *et al*., 1985; Tran *et al*., 1987). Since these pioneering studies, our understanding of the molecular mechanisms involved in these so-called clathrin-independent endocytic pathways has not progressed much. Paradoxically, it is the recent progress in the understanding of clathrin-dependent endocytosis that has made the largest contribution to our understanding of clathrin-independent endocytosis (see Figure 8.1) (Johannes and Lamaze, 2002). There is no longer any doubt about the existence and the importance of these alternative pathways, which constitute a major portal of entry for many toxins. After a brief summary of the main characteristics of each of the known endocytic systems operating in mammalian cells, we will examine how different toxins

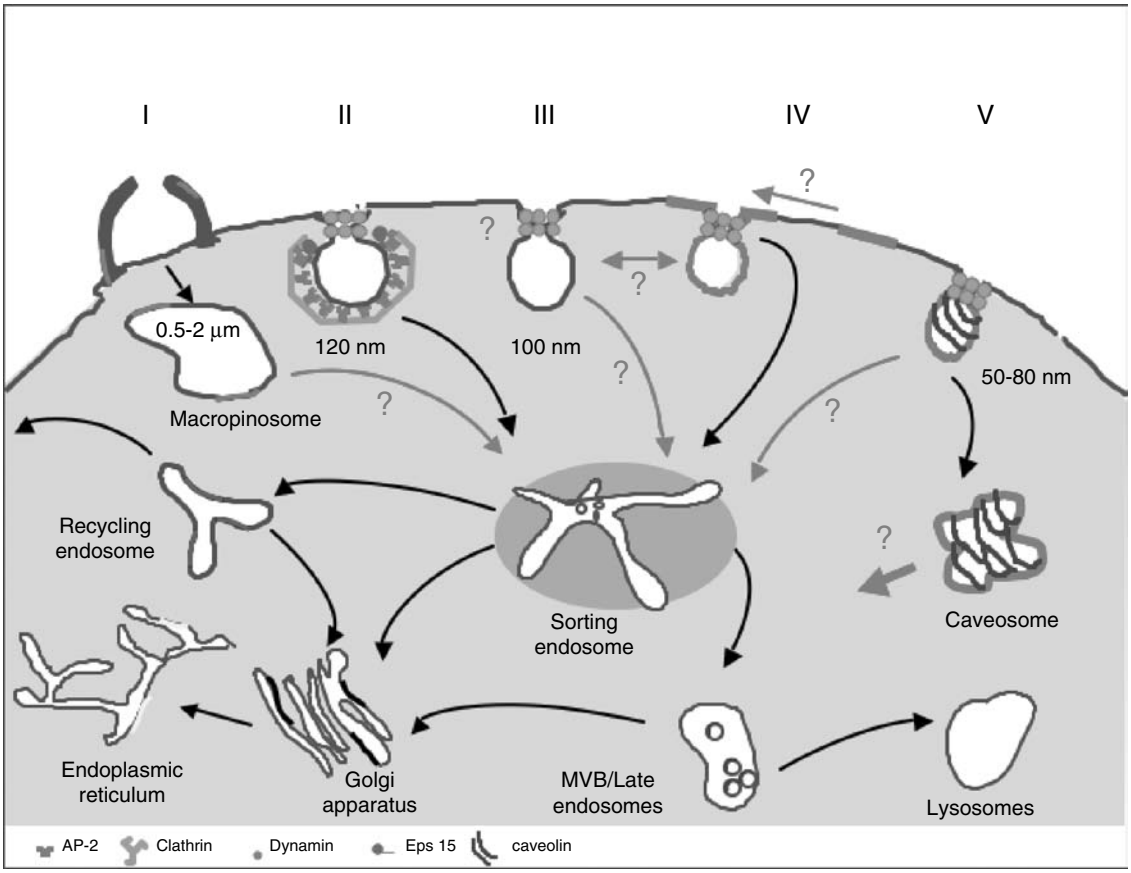



**FIGURE 8.1** Multiple endocytic pathways operate in mammalian cells. Besides the best-characterized clathrin-dependent endocytosis (II), several clathrin-independent endocytic pathways have been identified by molecular and morphological means and represent potential carriers for toxins delivery in the cell. Macropinocytosis (I) is a transient response to growth factors and mitogenic agents that contributes to fluid phase uptake through the formation of large vesicles of heterogeneous size. Non-coated invaginations (III) that pinch off to form smooth vesicles are commonly observed by conventional microscopy in different cell types and participate to fluid phase uptake. Certain lipid-based membrane microdomains including rafts (IV) and caveolae (V) are enriched in cholesterol and glycosphingolipids. Caveolin is an integral caveolar protein that gives a striated aspect to caveolae and allows us to distinguish them from other smooth invaginations. Caveolar endocytosis is mostly induced and can lead to the formation of "caveosomes" from which endocytosed molecules (SV40, CTx) could reach the endoplasmic reticulum and/or the nucleus. While it is not known whether lipid rafts tubulate or vesiculate to form smooth vesicles similar to (III), the vesicle budding GTPase dynamin is involved in clathrin-dependent endocytosis and some, but not all clathin-independent endocytosis. Black arrows represent established intracellular tracks whereas pathways with question marks have been less investigated (adapted from Johannes and Lamaze, 2002).

use these routes to enter cells. It should be noted, however, that most toxins can use several entry pathways and that endocytic plasticity is a trait common to many known toxins.

## CLATHRIN-DEPENDENT ENDOCYTOSIS

Clathrin-dependent endocytosis was first visualized in mosquito oocytes by electron microscopy 40 years ago (Roth and Porter, 1964). It is the oldest and the best-understood endocytic process in mammalian cells. Clathrin-dependent endocytosis is a constitutive process that begins with the concentration of high-affinity transmembrane receptors into specialized plasma membrane invaginations coated with clathrin (Brodsky *et al*., 2001; Owen *et al*., 2004; Rappoport *et al*., 2004). The coat proteins, clathrin and AP2, are the minimal machinery required to concentrate internalized molecules and to form a vesicle. The final step in clathrin-dependent endocytosis involves the dynamin GTPase, which is required for the release of cargo-loaded coated vesicles from the plasma membrane (Damke *et al*., 1994). Many other "accessory proteins" involved in the regulation of this process have been characterized in the last 10 years (Brodsky *et al*., 2001; Slepnev and De Camilli, 2000). Although it is classically thought that molecules internalized via clathrin-dependent endocytosis are either recycled to the plasma membrane or degraded via the late endosomal/lysosomal pathway, STx and CTx can be internalized through clathrin-coated pits and nevertheless escape the degradative pathway by going to the Golgi apparatus in a retrograde manner. These toxins can also use clathrin-independent endocytosis to enter the cells (see below). There are relatively few toxins that only undergo clathrin-dependent endocytosis. The best example is diphtheria toxin (DT), secreted by *Corynebacterium diphtheriae*. Morris *et al*. (1985) first showed that in the absence of functional coated pits, diphtheria toxin is unable to intoxicate Vero cells. Later work confirmed the entry of DTx by clathrin-mediated endocytosis and its trafficking into early endosomes where it translocates to its cytosolic target from acidic endosomes (Collier, 2001; Madshus *et al*., 1991; Simpson *et al*., 1998).

## CLATHRIN-INDEPENDENT PATHWAYS

The term *clathrin-independent endocytosis* includes a variety of endocytic mechanisms that are still poorly characterized at the molecular level and therefore are often described by negative properties. Classically, clathrin-independent endocytosis includes phagocytosis, macropinocytosis, and caveolar or non-caveolar lipid rafts (see Figure 8.1); (Johannes and Lamaze, 2002).

### Macropinocytosis and phagocytosis

Macropinocytosis is a ruffling-driven process that leads to the formation of large and irregularly sized endosomes in response to growth factors and other stimuli (Amyere *et al*., 2002). Macropinocytosis has never been shown to be involved in the entry of toxins *per se*, although it is likely that toxins such as ricin that bind to receptors present at most membrane sites (see above) are also present in macropinosomes together with general markers of endocytosis. Whether this entry can lead to a specific intracellular location or is associated with specific functions is not known. Macropinocytosis may play a more indirect role in the pathogenesis of toxin-secreting bacteria. For instance, toxins such as CNF1 secreted by *E. coli* activate Rho-GTPases, which in turn enhances macropinocytosis and therefore the uptake of pathogens (Steele-Mortimer *et al*., 2000). Phagocytosis, which allows the engulfment of solid particles by the cell, is not relevant to the entry of toxins and will not be discussed here.

### Caveolae

Although the role of caveolae in endocytosis is still debated, caveolae remain the best-characterized example of non-clathrin carriers present at the plasma membrane of many different cells. Their typical omega-like shape and their abundance in endothelial cells facilitated their identification. Indeed, they were identified in the early 1950s, almost 10 years before the discovery of clathrin-coated pits (Palade, 1953). These small (60 nm) plasma membrane invaginations are rich in cholesterol and sphingolipids, and represent a subset of lipid microdomains. Their particular lipid composition makes them sensitive to cholesterol depletion, a property shared with non-caveolar lipid rafts (see below) and to some extent with clathrin-coated pits (Rodal *et al*., 1999).

The structural and biological properties of caveolae are conferred by caveolin, a cholesterol-binding protein that distinguishes caveolae from other lipid microdomains. Caveolae have many functions, which have been extensively described in recent reviews. One of the most debated functions remains their contribution to clathrin-independent endocytosis (Nabi and Le, 2003; Parton and Richards, 2003; Pelkmans and Helenius, 2002). There are several reasons why the role

of caveolae in endocytosis is debated. First, in contrast with clathrin-coated pits, caveolin and morphologically detectable caveolae are not present in all cell types (Fra *et al*., 1994). Second, fluorescence recovery after photobleaching (FRAP) analysis of GFP-tagged caveolin has shown that caveolae are rather immobile structures at the plasma membrane (Thomsen *et al*., 2002), a property incompatible with an active role in constitutive endocytosis. However, recent studies using new approaches, such as expression of dominant negative mutants of caveolin or generation of caveolin-null mice, have provided new insight into the potential role of caveolae in endocytosis (Parton and Richards, 2003).

The complex role of caveolae in ligand uptake is best illustrated by the endocytosis of opportunistic ligands, such as viruses or toxins. Simian virus 40 (SV40) and CTx are the best-studied caveolar ligands, and both can be transported by caveolar endocytosis to caveosomes, a specialized subset of caveolin-1-positive endosomes with neutral pH (see below); (Pelkmans *et al*., 2001). CTx is a selective ligand for caveolar and non-caveolar lipid rafts, as shown by the sensitivity of its cytotoxicity to cholesterol-disrupting agents (Orlandi and Fishman, 1998; Wolf *et al*., 1998). Electron microscopy studies carried out in the early 1980s showed that gold-conjugated CTx bound to the GM1 glycolipid in smooth invaginations on the plasma membrane (Montesano *et al*., 1982; Tran *et al*., 1987). A few years later, it was shown that CTx did not bind uniformly in A431 cells and liver cells, and electron microscopy revealed the presence of caveolae. The presence of gold-conjugated CTx in early endosomes after warming the cells suggested that the toxin was internalized by caveolae (Parton, 1994). The finding that the dynamin GTPase, an actor of the clathrin-dependent machinery, was also associated with caveolae and required for their detachment further suggested that these organelles play a role in endocytic events (Henley *et al*., 1998; Oh *et al*., 1998). The endocytic potential of caveolae for toxins was also demonstrated by their ability to release CTx-loaded vesicles in a cell-free assay (Gilbert *et al*., 1999).

The fact that cells devoid of caveolae are still sensitive to the toxicity of CTx raised questions about the role of caveolae in CTx endocytosis and pathology (Nichols *et al*., 2001; Orlandi and Fishman, 1998; Torgersen *et al*., 2001). In the absence of caveolae, the internalization and toxicity of CTx relies on non-caveolar lipid microdomains because cholesterol depletion with methyl beta-cyclodextrin reversibly inhibits CT-induced chloride secretion and delays CTx endocytosis and toxicity (Wolf *et al*., 2002). Another study suggested a link between toxicity and sorting at the plasma membrane (Wolf *et al*., 1998). In this work, the authors compared the requirements for the toxicity of CTx and the closely related LTx enterotoxin from *E. coli*. Whereas CTx, which binds to GM1 in intestinal epithelial cells, was present in biochemically defined rafts, LTx, which binds to the glycolipid GD1a, was found in non-raft fractions. Although both toxins bear similar catalytic subunits, only raft-associated CTx was able to elicit a cAMP response. Whether this differential effect on toxicity is due to raft-dependent differences in trafficking or in signaling is not known (see below). Another layer of complexity is added by the possibility for CTx to be internalized by clathrin-coated pits in several cell types (Massol *et al*., 2004; Nichols, 2002; Shogomori and Futerman, 2001; Torgersen *et al*., 2001). The entry of CTx through clathrin-dependent endocytosis is consistent with the presence of GM1 in clathrin-coated pits (Parton, 1994). It should be pointed out that the natural distribution of the GM1 glycolipid is likely to be modified by the multivalence of CTx, as toxin-induced relocalization of GM1 to caveolar microdomains has been observed (Harder *et al*., 1998; Spiegel *et al*., 1984). Electron microscopy examination of cell sections after low-temperature embedding revealed a weaker concentration of GM1 in caveolae than in clathrin-coated pits. In contrast, CTx binding to GM1 induced the redistribution of GM1 to caveolae (Parton, 1994). These data were recently confirmed by fluorescence resonance energy transfer (FRET) on living cells, showing that CTx-induced GM1 clusters were excluded from clathrin-coated pits (Nichols, 2003).

Whereas these results question the role of clathrin-coated pits in the uptake of CTx, other studies point out some cell type specificities. For example, in hippocampal neurons, CTx bound to GM1 was found in lipid microdomains and Ctx endocytosis was blocked by inhibitors of clathrin-dependent endocytosis, whereas filipin and cyclodextrin, two compounds known to destabilize these microdomains, had no effect (Shogomori and Futerman, 2001). These authors proposed that CTx is internalized either through clathrin-coated pits after escaping from lipid rafts or directly after the diffusion of lipid rafts into clathrin-coated pits. This model was recently validated for the uptake of the anthrax toxin (see below). Finally, the endocytic "equipment" of the cell may affect toxin sorting at the plasma membrane. For example, CtxB is internalized via clathrin-independent endocytosis in cells with high caveolin-1 expression, whereas significant clathrin-dependent uptake occurs in cells with low caveolin-1 expression (Singh *et al*., 2003). This study also showed that fluorescent GM1, normally internalized by clathrin-independent endocytosis in HeLa cells with low caveolin-1 expression, is partially

internalized via the clathrin pathway in the presence of CTx. Accordingly, several studies have shown that caveolin-1 overexpression inhibits rather than stimulates clathrin-independent endocytosis (Le PU *et al*., 2002; Minshall *et al*., 2000; Sharma *et al*., 2004a). Likewise, the amount of GM1 present at the plasma membrane can determine the route by which CTx is endocytosed; CTx endocytosis is sensitive to cholesterol depletion when high levels of GM1 are expressed, whereas cholesterol depletion has a minor effect in cells expressing low levels of GM1 (Pang *et al*., 2004).

These results suggest that CTx can switch internalization pathways according to the number of caveolae and/or the amount of GM1 present at the plasma membrane. However, the role of this endocytic plasticity and, more particularly, of clathrin-dependent endocytosis in the toxicity of CTx remain to be determined. The complex relationship between CTx and endocytosis has not yet been resolved totally because Parton's group recently characterized a third pathway for the uptake of CTx that involves some new endocytic carriers other than clathrin-coated pits and caveolae (see below) (Kirkham *et al*., 2005).

STx, which, like CTx, relies on glycolipid binding for its uptake and toxicity, has also been shown to enter host cells through clathrin-independent endocytosis (Nichols *et al*., 2001; Puri *et al*., 2001; Saint-Pol *et al*., 2004) (see below). Although the Gb3 ceramide (CD77), the STx gycolipid receptor, has been found associated with Triton X-100-resistant membranes in HeLa cells (Falguières *et al*., 2001), it is not known whether these microdomains include caveolae and whether caveolae are involved in the uptake of STx.

## Caveolae-independent mechanisms

Caveolae are just one example of the cholesterol-rich microdomains found in the plasma membrane. There is an increasing amount of evidence supporting the raft hypothesis, i.e., the organization of the plasma membrane into sphingolipid- and cholesterol-enriched microdomains (Brown and London, 2000; Johannes and Lamaze, 2002; Sharma *et al*., 2002; Simons and Ikonen, 1997). A recent study has shown that microdomains of the raft type are very small (within the nanometer range) and highly dynamic structures that are capable of self-assembly for specific sorting of membrane proteins and glycolipids (Sharma *et al*., 2004b). Although the functional role of lipid-based membrane microcompartmentalization is currently a subject of intense debate (Munro, 2003) and much work is still needed to characterize these membrane structures, there is evidence suggesting a role for noncaveolar rafts in endocytosis (Johannes and Lamaze, 2002; Lamaze *et al*., 2001; Nabi and Le, 2003; Sharma *et al*., 2002). Due to their small size, it has been suggested that lipid rafts can be internalized by any endocytic vesicles.

This is best illustrated by the interleukin-2 receptor, which is also associated with lipid microdomains and is internalized in a clathrin- and caveolin-independent manner (Lamaze *et al*., 2001). This pathway still shares some mechanistic similarities with caveolar and clathrin-dependent endocytosis because IL-2 uptake requires the activity of the dynamin GTPase. A similar clathrin- and caveolae-independent endocytic pathway was recently described for the uptake of glycosylphosphatidylinositol (GPI)-anchored proteins through so-called GEECs (GPI-AP-enriched early endosomal compartments) (Sabharanjak *et al*., 2002). In this case, however, endocytosis of GFP-tagged GPI occurs via a dynamin-independent and CDC42-regulated pathway. Whether some toxins can also follow the interleukin-2 pathway is not yet known, but recent data have shown that CTx can enter cells through the GPI-AP pathway (see below). Another entry mechanism was recently characterized for the signaling and the internalization of immune receptors, including the T cell and the B cell receptors (BCR) (Crotzer *et al*., 2004; Dykstra *et al*., 2001; Stoddart *et al*., 2002). These authors suggested that BCR uptake occurs in different B cell lines only when clathrin is associated with rafts and is tyrosine phosphorylated by the Src kinase upon cross-linked BCR-induced association with lipid rafts.

The anthrax toxin is another example of a toxin that is taken up via clathrin-coated pits and lipid rafts. In this case, however, both pathways must be coordinated for the efficient entry of the toxin and intoxication of the host cell (Abrami *et al*., 2003; Kurzchalia, 2003). As described earlier, it is only after processing PA83, the so-called anthrax receptor (ATR) (Bradley *et al*., 2001), by furin family proteases that PA63 forms heptamers, allowing the entry of EF and LF (Cunningham *et al*., 2002; Petosa *et al*., 1997). Abrami and collaborators (Abrami *et al*., 2003) provided interesting new insight into the role of lipid rafts in the entry of the cleaved form of PA83. The major finding of this work is that ATR bound to heptameric PA63 but not to monomeric PA83 is associated with biochemically defined lipid rafts, i.e., detergent-resistant membranes. It appears that oligomerization of PA63 is essential, as artificial antibody-induced cross-linking of PA83 also leads to the association with lipid rafts. When cross-linked and associated with lipid rafts, the complex is internalized more efficiently than monomeric PA83. Although the association with lipid rafts is consistent with cholesterol-dependent entry, such as caveolae, the use of selective inhibitors of clathrin-dependent endocytosis

revealed that the PA63/EF/LF complex is internalized by clathrin-dependent endocytosis. In this context, the endocytic properties of anthrax toxin are similar to what was described for the B cell and the T cell receptors. As already mentioned, CTx may be taken up through a similar process in neuronal cells (Shogomori and Futerman, 2001). Whether ligand-induced oligomerization of anthrax or CTx also requires the activity of the src kinase, as described for these receptors, is not known. These examples reveal the tight interplay between lipid rafts and the clathrin machinery, and the complex nature of the endocytosis of toxins.

The observation that STx, which also binds raft-associated Gb3, can be internalized through clathrin-coated pits (Sandvig *et al*., 1989) raises the possibility that STx uptake can occur by one of the above-mentioned mechanisms. However, recent evidence does not support a model in which Gb3-bound STx is first associated with rafts and then internalized through clathrin-coated pits as described for the anthrax toxin. Indeed, a detailed analysis of the role of clathrin in STx internalization and intracellular transport has shown that STx is taken up normally in cells where clathrin function was inhibited (Saint-Pol *et al*., 2004). These data suggest that STx can enter cells by clathrin-dependent and/or -independent pathways. Whether clathrin-independent endocytosis operates in response to the inhibition of clathrin-dependent endocytosis, as shown for fluid phase markers in HeLa cells (Damke *et al*., 1995), or coexists with clathrin-dependent endocytosis is not known. Likewise, the contributions of each pathway in the overall uptake of STx and cell toxicity and the role of Gb3 binding in targeting each pathway have not yet been elucidated.

The endocytosis of STx appears to be independent from the dynamin GTPase (Lauvrak *et al*., 2004), a characteristic that distinguishes STx uptake from that of the IL-2 receptor and to a lesser extent from CTx internalization because CTx uptake is reduced by 50% in dynamin-inactivated cells (Torgersen *et al*., 2001). Most of the studies on clathrin-independent endocytosis used non-selective pharmacological inhibitors; this lack of selectivity has hampered the thorough molecular characterization of the alternate pathways (Johannes and Lamaze, 2002). Likewise, very few ultrastructural studies have addressed the morphological properties of these alternative pathways. Recently, however, Parton's group used quantitative light microscopy and a modified immunoelectron microscopic technique to characterize the entry pathway of CTx in primary embryonic fibroblasts (Kirkham *et al*., 2005). While this study showed that CTx can be internalized by clathrin-coated pits and caveolae, it indicated that CTx was rather trapped in caveolae, supporting the static role of caveolae previously demonstrated in some cell types (Thomsen *et al*., 2002). More importantly, this study revealed that 50% of CTx was efficiently transported through some uncoated tubular or ring-shaped structures also containing GPI-anchored proteins and fluid phase markers. Interestingly, the molecular characterization of this pathway revealed that the novel caveolae- and clathrin-independent tubular/ring-like carriers identified in these cells are indeed the same structures, GEECs, shown to be involved in the cdc42-dependent uptake of GPI-APs and fluid phase markers described earlier in this review (Sabharanjak *et al*., 2002). The particular role of this pathway in the cytotoxicity of CTx has not yet been documented. It will be interesting to determine whether STx, which shares many characteristics with CTx, including dynamin-independent entry and dynamin-dependent endosome to Golgi transport (Lauvrak *et al*., 2004), can also use this pathway.

About 50% of so-called fluid phase endocytosis would occur in the absence of functional clathrin-coated pits or in dynamin-inactivated cells (Cupers *et al*., 1994; Damke *et al*., 1995). It is likely that fluid phase endocytosis encompasses more examples of pinocytic uptake than the dynamin-independent pathways described above and that other, as yet uncharacterized, pinocytic pathways exist in mammalian cells. The uptake of the CNF1 toxin might occur via one of these uncharacterized pathways. Thus, in Hep-2 cells, the cytotoxicity of CNF1 is not blocked by filipin, a compound that reduces cholera toxin internalization by the caveolae-like mechanism (Contamin *et al*., 2000). Furthermore, Hep-2 cells, which contain proteins that inhibit the formation of clathrin-coated vesicles, are still sensitive to CNF1. After internalization by both clathrin- and caveolae-like-independent pathways, CNF1 is subsequently transferred to an endosomal compartment by a microtubule-dependent mechanism. CNF1 requires an acidic cell compartment to transfer its enzymatic activity into the cytosol in a manner similar to that required by diphtheria toxin. CNF1 is therefore the first bacterial toxin described that uses both a clathrin-independent endocytic mechanism and an acid-dependent membrane translocation step for delivery of the catalytic domain to the cytosol.

## INTRACELLULAR DISTRIBUTION PATHWAYS

Although numerous toxins, e.g., cholera and Shiga toxin, can enter cells by clathrin-dependent and independent mechanisms (see above), most of them are subsequently found in the transferrin receptor (TfR)-positive

"conventional" early endosome (EE). There are several subsequent sorting opportunities. Some toxins, such as ricin, recycle to the plasma membrane. Others, such as anthrax toxin, are efficiently targeted to the late endocytic pathway. Others, such as Shiga toxin, avoid recycling and degradation to reach other compartments in the cell using a recently discovered pathway termed the *retrograde route*. These different pathways are discussed in detail below (see also Figure 8.2).

## RECYCLING OF TOXINS TO THE PLASMA MEMBRANE

Proteins and lipids can recycle from endosomes back to the plasma membrane. Within the early endocytic pathway, two routes have been described using TfR as a prototypic recycling marker. In association with its receptor, some internalized Tf very rapidly returns to the plasma membrane with halftimes of around two minutes, depending on cell type. Another fraction remains associated with the cells longer and returns to the plasma membrane with halftimes of around 12 minutes. This slow recycling material is found in the pericentriolar region of many cells, often referred to as the *recycling endosome* (RE). As no general consensus currently exists on the terminology used to describe the early endocytic pathway, we will define the membrane structures that constitute the pathway.

The vacuolar organelles that result from the fusion of endocytic vesicles are often termed *early* or *sorting endosomes*. It is from these endosomes that recycling receptors return to the plasma membrane with very short halftimes (Mukherjee *et al*., 1997). Tubular membrane projections detach from these vacuoles, which, as part of their transformation process, start forming internal vesicles, the so-called multivesicular endosomes (Gruenberg and Stenmark, 2004). The tubular elements are often, but not always (for example, HeLa cells) (Lin *et al*., 2002), concentrated in the pericentriolar area, where they are called *RE*, due to the localization of recycling receptors to these structures. As mentioned above, recycling from RE is slower than recycling from peripheral early or sorting endosomes. There is some debate about whether the RE is an independent organelle. Considering: (i) that no single marker protein that can be used to distinguish RE from early or sorting endosomes has yet been described; (ii) that they recycle proteins to the plasma membrane in a similar way to early or sorting endosomes; (iii) and that it is likely that other sorting processes such as retrograde sorting to the TGN already start on early or sorting endosomes and continue on RE (see below), we prefer to consider the early endocytic membranes as one network and name them the *early/recycling endosomes* (EE/RE).

The mechanisms of sorting in the early endocytic pathway are still poorly understood. There is a general consensus that areas of high membrane-to-volume ratios, i.e., tubular structures, allow the retrieval of many receptors from vacuolar early or sorting endosomes that mature to become multivesicular late endosomes. Protein toxins are associated with membrane receptors, meaning they are prone to recycling. This is well documented for ricin (McIntosh *et al*., 1990). However, membrane association does not necessarily cause recycling. STx, for example, does not return to the plasma membrane (Mallard *et al*., 1998; Schapiro *et al*., 1998). How recycling is prevented is not yet known, but it is possible that association with raft type membrane microdomains is involved (see below).

## TOXIN TRAFFICKING TO LATE ENDOSOMES/ LYSOSOMES

A number of protein toxins use the endocytic pathway to reach a low pH environment that favors the translocation of their catalytic subunits to the cytosol. These toxins, such as diphtheria and anthrax toxins, are discussed in detail elsewhere in this book. A critical question is at what stage of the endocytic pathway these toxins cross membranes to release their catalytic chain into the cytosol. Several lines of evidence suggest that early endosomes are the site of translocation for the diphtheria toxin (Lemichez *et al*., 1997; Papini *et al*., 1993). Recent studies have shed new light on how endosomal subcompartmentalization can influence anthrax toxin translocation.

This work was made possible by the discovery of new elements of the endosomal sorting machinery that are involved in the formation of internal vesicles, the multivesicular endosomes (Gruenberg and Stenmark, 2004). One of these elements is the ALIX protein, which is involved in the regulation of the dynamics of late endosomal internal vesicles (Matsuo *et al*., 2004). The other element is a lipid, lysobisphosphatidic acid, antibodies to which also affect this process (Kobayashi *et al*., 1998). Interfering with ALIX or lysobisphosphatidic acid has distinct effects on the membrane translocation of diphtheria toxin and anthrax toxin, consistent with the following models (Abrami *et al*., 2004). Anthrax toxin is trapped in internal vesicles within multivesicular endosomes, and the catalytic lethal factor is transferred into the intravesicular lumen, the topology of which is that of the cytosol. Back-fusion of

the internal vesicles with the limiting membrane of multivesicular endosomes at late stages of the endocytic pathway leads to secretion of the lethal factor into the cytosol and allows it to access its cytosolic signaling targets. In contrast, diphtheria toxin is concentrated around the limiting membrane, and membrane translocation starting at the level of the early endosome enables direct transfer of the catalytic fragment to the cytosol.

## THE RETROGRADE ROUTE

Some protein toxins do not depend on endosomal acidification for their translocation to the cytosol. These include inhibitors of protein biosynthesis that modify ribosomal RNA, such as STx; the related verotoxins (or Shiga-like toxins); the plant toxin ricin; and *Pseudomonas* exotoxin (see Figure 8.2). They also include CTx, which induces ADP ribosylation of Gsα. These toxins escape from the endocytic pathway to be targeted to the endoplasmic reticulum (ER). Several studies have strongly suggested that it is from the ER that the catalytic subunits of these toxins are transferred to the cytosol (Simpson *et al*., 1999; Tsai *et al*., 2001; Wesche *et al*., 1999), using the cellular retrotranslocation or ERAD machinery (Tsai *et al*., 2002). After endocytosis, the retrograde route comprises two key steps: transport from endosomes to the TGN/Golgi apparatus and transport from the TGN/Golgi apparatus to the ER. The following sections will deal with each of these steps.

### Endosome-to-TGN transport

The first pathway to be described between the endocytic and the biosynthetic/secretory routes was the late endosome-to-TGN pathway followed by the mannose

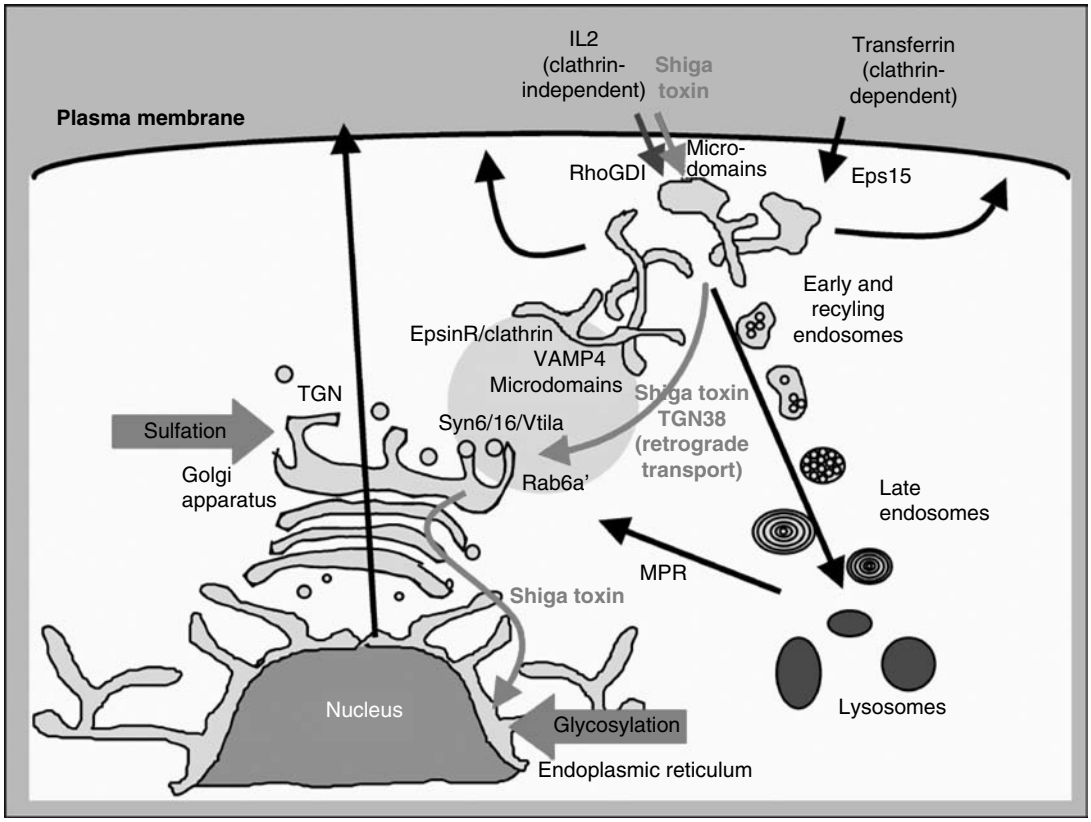



**FIGURE 8.2** Intracellular transport pathways of Shiga toxin. Black arrows indicate the classical transport pathways. Shiga toxin enters cells at least in part through clathrin-independent endocytosis. The gray circle represents the interface between early/recycling endosomes and the trans-Golgi network. Shiga toxin reaches this interface and is then transported to the Golgi apparatus via the retrograde pathway (gray arrow). The retrograde pathway is distinct from the well-known recycling pathway used by the mannose-6-phosphate receptor (MPR), even though recent evidence suggests that the MPR may also use the early/recycling endosomes-TGN interface. Shiga toxin is then transported from the Golgi apparatus to the endoplasmic reticulum (gray arrow). RhoGDI and Eps15 are specific inhibitors of the indicated endocytic pathways. Sulfation and glycosylation on corresponding B-subunit mutants are used to detect and quantify the arrival of the B-subunit in the trans-Golgi network and the endoplasmic reticulum, respectively. At the early endosome/TGN interface, selected molecular players are shown that have been directly or indirectly implicated in this transport step (See text for other retrograde sorting factors; adapted from Johannes, 2002).

6-phosphate receptor (see (Ghosh *et al*., 2003) (Figure 8.2). The molecular hallmarks of this pathway are the GTPase Rab9 (Lombardi *et al*., 1993) and the putative coat component TIP47 (Diaz and Pfeffer, 1998). To date, none of these has been shown to be involved in toxin transport (Lauvrak *et al*., 2002; Simpson *et al*., 1995), with the possible exception of CTx (Choudhury *et al*., 2002).

STx was particularly instrumental for the discovery of an alternative transport pathway between compartments of the early endocytic pathway and the TGN: the early/recycling endosome-to-TGN route (Johannes and Goud, 1998; Lord and Roberts, 1998; Sandvig *et al*., 2004) (Figure 8.2). In cells that are sensitive to STx-dependent inhibition of protein biosynthesis, it has been found that very little STx is recycled to the plasma membrane (Mallard *et al*., 1998; Schapiro *et al*., 1998) and that STx is absent from compartments of the late endocytic pathway (Mallard *et al*., 1998). Instead, the protein extensively overlaps with markers of early/recycling endosomes, such as the TfR. The toxin is then rapidly transferred from early/recycling endosomes to the TGN and the cisternae of the Golgi apparatus.

Molecular studies with quantitative methods, such as protein sulfation and glycosylation using engineered Shiga toxin B-subunit (STxB) variants (Johannes *et al*., 1997; for a review, see Mallard and Johannes, 2002), reconstitution approaches, and morphological tools have confirmed the existence of an EE/RE-to-TGN transport pathway (Figure 8.2). It has been shown that EE/RE-to-TGN transport involves a TGN-localized tSNARE complex composed of the heavy chain tSNARE syntaxin 16, the light chain tSNAREs Vti1a and syntaxin 6, and of two early/recycling endosomal vSNAREs, VAMP3/cellubrevin, and VAMP4 (Mallard *et al*., 2002). Both vSNAREs form independent complexes with the tSNAREs. A role for the trans-Golgi/TGN localized GTPase Rab6a' in retrograde transport was described in the same study. Upon expression of dominant negative Rab6a' mutants, STxB accumulates in the EE/RE. Rab6a' plays a predominant role in EE/RE-to-TGN transport, whereas other TGN or endosome-localized GTPases, such as Rab11 (Wilcke *et al*., 2000) and Rab9 (see above) appear to have, respectively, ill-defined or no functions.

The availability of quantitative and morphological STxB-based tools for the study of retrograde transport has allowed the biochemical dissection of EE/RE-to-TGN transport. In one study, another SNARE complex composed of syntaxin 5, GS15, GS28, and Ykt6 was found to be involved in STx transport from the EE/RE to the TGN (Tai *et al*., 2004). This is surprising given the function of syntaxin 5 in trafficking at the interface between the ER and the cis-Golgi (Dascher *et al*., 1994). However, other studies detected syntaxin 5 deep within Golgi stacks and in several SNARE complexes (Hay *et al*., 1998). These results suggest that syntaxin 5 functions in trafficking at several sites. They also show that trafficking at the EE/RE-TGN interface is more complex than initially anticipated, and that several molecular machineries may co-exist for the same transport intermediates, or that parallel pathways (the functions of which remain to be elucidated) exist.

The complexity of retrograde trafficking is further illustrated by considering molecular coats at the EE/RE-TGN interface. Clathrin was the first coat for which the function was addressed at this interface. Indeed, in addition to its well-established presence at the plasma membrane and the TGN, clathrin was found in endosomes (Stoorvogel *et al*., 1996). Cryo-electron microscopy demonstrated clathrin and the clathrin adaptor AP1 on the STxB-containing EE/RE (Mallard *et al*., 1998; Saint-Pol *et al*., 2004), and functional experiments confirmed a role for clathrin in STxB transport to the TGN (Lauvrak *et al*., 2004; Saint-Pol *et al*., 2004). Unexpectedly, interfering with AP1 function had no effect, even though several studies suggested a role for AP1 in retrograde transport (Folsch *et al*., 2001; Mallard *et al*., 1998; Meyer *et al*., 2000; Valdivia *et al*., 2002). Instead, the clathrin adaptor epsinR, a phosphatidylinositol 4-phosphate-binding protein, was found on the STxB-containing EE/RE and shown to be involved in retrograde sorting (Saint-Pol *et al*., 2004).

Another coat with functions at the EE/RE-TGN interface is the retromer. In yeast, this five-protein complex is involved in retrieval of receptors from the vacuole (Seaman *et al*., 1998). Recent studies on mammalian cells have suggested that this function is conserved at the EE/RE-TGN interface (Arighi *et al*., 2004; Seaman, 2004). Whether retromer is also involved in toxin trafficking and how clathrin and retromer operate in the same or parallel pathways remain to be elucidated.

Retrograde trafficking of STxB is also controlled by GPP130, a protein of unknown function (Natarajan and Linstedt, 2004), the GRIP domain protein Golgin-97 (Lu *et al*., 2004), and dynamin (Lauvrak *et al*., 2004).

Although studies on STxB led to the discovery of the first molecular players at the EE/RE-TGN interface, other toxins have also been used. For example, ricin has been used to study the role of dynamin (Llorente *et al*., 1998), cholesterol (Grimmer *et al*., 2000), calcium (Lauvrak *et al*., 2002), and protein kinase A (Birkeli *et al*., 2003). The problem with ricin as a cell biology tool is that only 5% of the cell-associated toxin is transported back to the TGN/Golgi apparatus (van Deurs *et al*., 1988), in association with one or several unidenti-

fied receptors. Thus, it is difficult to judge whether an inhibitory effect on retrograde transport, such as with dynamin, arises due to specific perturbation of retrograde transport-competent receptor trafficking at the plasma membrane or at any other step of the retrograde route.

Too little comparative information is currently available to determine whether the protein toxins that use the retrograde route (STx, CTx, ricin, etc.) depend on the same molecular machineries. The only molecular tools that have been applied to several toxins are dynamin, Rab9, and Rab11. Rab9 has no effect (see above). Rab11 and dynamin have similar effects on the retrograde sorting of STx and ricin (Iversen *et al*., 2001; Lauvrak *et al*., 2004; Llorente *et al*., 1998; Wilcke *et al*., 2000). As for Rab11, dominant negative or active mutants produced phenotypes that were relatively weak and difficult to interpret. Therefore, more studies are required to come to firm conclusions.

The retrograde route used to transport protein toxins between the EE/RE and the TGN is pre-existing and required for the trafficking of several cellular proteins. A detailed discussion of these examples would go beyond the scope of this review. The mannose 6-phosphate receptor, a shuttling protein required for the transport of mannose 6-phosphate-modified enzymes to lysosomes, uses this interface (see Saint-Pol *et al*., 2004 and references therein), in addition to the late endosome-to-TGN route. Several ubiquitous proteins with unknown functions have been mapped to the retrograde route: TGN38/46 (Ghosh *et al*., 1998; Mallard *et al*., 2002), GPP130, and GP73 (Natarajan and Linstedt, 2004). Upon insulin stimulation, the glucose transporter in adipocytes and muscle cells, Glut4, shuttles between an internal site and the plasma membrane, resulting in dynamic control of its activity; this internal site appears to be connected to the retrograde route (Shewan *et al*., 2003). Viruses (Bantel-Schaal *et al*., 2002) and viral proteins (Blot *et al*., 2003; Crump *et al*., 2004) have been described to reach the Golgi apparatus from the plasma membrane, possibly by EE/RE-to-TGN transport. Finally, infectious prion proteins might undergo retrograde transport to the ER (Beranger *et al*., 2002), followed by retrotranslocation to the cytosol (Ma and Lindquist, 2001).

Proteins are not the only determinants for retrograde transport. Indeed, CTx and STx are both associated with so-called membrane microdomains, or "rafts" (Harder *et al*., 1998; Katagiri *et al*., 1999), and in both cases, there is evidence suggesting a role for lateral lipid asymmetry in retrograde sorting. In the case of CTx, a related toxin, LTIIb, binds to the glycosphingolipid GD1a but not to GM1. In epithelial cells, CTx, but not LTIIb, is raft associated. This difference correlates with differences in their intracellular trafficking, CTx being able to enter the retrograde route, but not LTIIb (Fujinaga *et al*., 2003). Similar conclusions about the role of membrane microdomains in sorting at the EE/RE-TGN interface have been reached for STx based on the comparison of STx transport in different cell types. Unlike HeLa cells (Eiklid and Olsnes, 1980), monocytes and monocyte-derived cells, i.e., macrophages and dendritic cells, are resistant to STx (Ramegowda and Tesh, 1996; Tesh *et al*., 1994; van Setten *et al*., 1996). A correlation has been observed between this differential sensitivity to STx and the trafficking of the toxin in the respective cell types (Falguières *et al*., 2001). While STx follows the retrograde route in HeLa cells, no targeting to the TGN/Golgi or the ER can be detected in monocyte-derived cells. Interestingly, STx has been found in detergent-resistant membranes (DRMs) in HeLa cells, but not in monocyte-derived cells, suggesting that DRM association is required for retrograde sorting. Indeed, STx is associated with DRMs throughout its retrograde transport to the ER, and retrograde transport of STx is inhibited when microdomains are reversibly destabilized in HeLa cells. The mechanisms by which membrane microdomains contribute to the retrograde sorting of toxins remain to be determined.

Receptor heterogeneity has also been shown to be involved in retrograde transport of STx to the ER. The presence of Gb3 with C16 and C18 fatty acid chains was correlated with retrograde transport and intoxication of cells (Arab and Lingwood, 1998; Sandvig *et al*., 1996). However, this correlation has not yet been demonstrated directly. The ricin receptor involved in retrograde transport is still unknown. It does not seem to be a glycosphingolipid, however, as cells without glycosylceramide synthase activity are still normally sensitive to the toxin (Spilsberg *et al*., 2003).

## TGN/Golgi-to-ER transport

After reaching the TGN, protein toxins like STx, CTx, and ricin need to be targeted to the ER to reach the retrotranslocation machinery. The most direct evidence for this is based on experiments using the fungal metabolite brefeldin A, which redistributes Golgi markers into the ER, blocking anterograde and retrograde transport at the level of the biosynthetic/secretory pathway. In the presence of brefeldin A, cells are protected from STx (Donta *et al*., 1995), CTx (Nambiar *et al*., 1993), ricin, and *Pseudomonas* exotoxin (Yoshida *et al*., 1991). Quantitative measurements showed that this protection is due to the efficient blockage of retrograde transport (Mallard *et al*., 1998). However, cells are not totally protected by brefeldin A treatment,

indicating that at higher concentrations, toxins may escape from endosomes to the cytosol (Beaumelle *et al*., 1993).

Some proteins can follow the biosynthetic/secretory pathway in the opposite direction. This is the case for resident proteins of the ER that are retrieved from Golgi cisternae after escaping from their steady-state localization in the ER (Pelham, 1996). The retrieval signals are basic amino acids found at the C- or N-termini of type I or type II trans-membrane proteins, respectively, or the KDEL tetrapeptide at the C-terminus of luminal proteins. When retrieval signal-tagged resident ER proteins encounter sorting receptors in the intermediate compartment or in Golgi cisternae up to the TGN (Miesenbock and Rothman, 1995), their retrograde transport to the ER is induced. The retrograde sorting machinery at the Golgi-ER interface includes components of the COPI coat, which interact directly with retrieval signals, and mutation of which has been correlated with retrieval phenotypes (for a review, see Cosson and Letourneur, 1997).

With respect to toxins, two central questions arise. Do they cross the stacks of the Golgi apparatus en route to the ER: Is their retrograde transport dependent on the "classical" retrieval machinery involving the KDEL receptor and the COPI coat?

To date, no definite answer exists for the first question. Analysis of the retrograde transport of STx by immunofluorescence and immunoelectron microscopy revealed that the toxin first accumulates in the TGN and then very rapidly enters the Golgi stacks (Mallard *et al*., 1998), before becoming detectable in the ER (Kim *et al*., 1998). Furthermore, when membrane trafficking at the ER-Golgi interface is disrupted by using mutants of the gamma-tubulin and Golgi-binding protein GMAP210, STx accumulates in Golgi cisternae (Pernet-Gallay *et al*., 2002). These data are consistent with the notion that STx can reach the ER via Golgi cisternae. They do not exclude, however, the possibility that toxins might also be targeted directly from the TGN to the ER (Storrie *et al*., 2000).

The second question concerns the role of the KDEL-receptor/COPI machinery in retrograde TGN/Golgi-to-ER trafficking of toxins. Indeed, some toxins have KDEL signals, and there is evidence that their toxicity depends on these signals (Johannes and Goud, 1998). However, the observed effects were quite weak, and not consistent with the hypothesis that "conventional" retrieval signals play a critical role in toxin trafficking. This question was thus addressed more directly in a series of experiments on STx, CTx, PEx, and ricin.

STx does not carry a KDEL signal. Two approaches have been used to test whether the KDEL-receptor/COPI machinery was nevertheless involved in its trafficking to the ER. The first approach involved interfering with KDEL-receptor activity. This did not protect cells against STx (Jackson *et al*., 1999), and did not prevent STx from reaching the ER (Girod *et al*., 1999). It was suggested that the KDEL-receptor/COPI independent transport of STx to the ER involves the GTPase Rab6 (Girod *et al*., 1999; White *et al*., 1999), but a detailed molecular description of this route is still lacking. It is noteworthy that this transport step is also used by endogenous proteins. Indeed, if anterograde transport out of the ER is inhibited, Golgi markers accumulate in the ER, and this accumulation is not inhibited by interfering with KDEL-receptor/COPI function (Girod *et al*., 1999).

The second approach involved adding an ectopic KDEL retrieval signal to the C-terminus of STxB. Quantitative biochemical assays showed that this modification had no effect on retrograde transport of the protein to the ER (Johannes *et al*., 1997). The KDEL signal did, however, lead to an efficient retention of STxB in the ER. Thus, it appears that the minor effects of retrieval signals on protein toxin activities are explained, not by stimulating their retrograde transport as such, but by increasing the effective toxin concentration in the ER, thereby promotingretrotranslocation.

This proposal was addressed in a recent series of experiments on CTx and LTx. Both of these carry retrieval signals on their catalytic A-subunits, either the canonical KDEL signal in the case of CTx, or a variant (HDEL) in the case of LTx. In agreement with the abovementioned data on STxB and using similar quantitative biochemical tools, it was found that CTxB is transported to the ER with high efficiency, and independently of the presence of KDEL-bearing CTxA (Fujinaga *et al*., 2003). Mutating the retrieval signals on CTx or LTx poorly reduced toxicity (Lencer *et al*., 1995). Based on both the trafficking and toxicity data, it is clear that it is not the arrival in the ER that depends on the retrieval signals, but retention in this compartment and subsequent retrotranslocation. Such retention might be of particular importance in the case of CTx, as its A and B subunits dissociate before membrane translocation (Bacia *et al*., 2002; Bastiaens *et al*., 1996; Majoul *et al*., 1996), requiring retention of the non-membrane bound A-subunit from secretion by bulk flow.

This conclusion is further confirmed by the following observations. Impairment of COPI function using drugs had no effects on intoxication of cells by CTx (Chen *et al*., 2002a). However, morphological experiments have shown that microinjection of interfering anti-COPI or anti-KDEL receptor antibodies leads to the accumulation of CTxA in the Golgi apparatus (Majoul *et al*., 1998). Again, the most straightforward interpretation is to assume that arrival in the ER is not

impaired, explaining the efficient intoxication. However, once in the ER, CTx starts cycling between the ER and Golgi. When KDEL-bearing CTx encounters the KDEL receptor, which re-localizes to Golgi cisternae when COPI is dysfunctional, its localization is also shifted to this compartment.

The retrograde transport of ricin to the ER is COPI independent (Chen *et al*., 2003). Furthermore, toxins have been used to show that Golgi-to-ER transport is actin- (Duran *et al*., 2003; Luna *et al*., 2002; Valderrama *et al*., 2001) and calcium- (Chen *et al*., 2002b) dependent.

## NON-CONVENTIONAL ENDOSOMAL PATHWAYS

The sections above are based on the "conventional" model of the endocytic pathway being composed of early/recycling endosomes and late endosomes/lysosomes. Recent studies suggested that the complexity of structures may be higher than described by the "conventional" model, particularly at the level of the early endocytic pathway. The best-characterized example of these structures is the caveosome. This term was coined by the Helenius group studying the internalization of SV40 (Pelkmans *et al*., 2001). Following complex events at the plasma membrane involving actin remodeling (Pelkmans *et al*., 2002), SV40 is internalized into caveolin-positive membranes that are devoid of TfR and Rab5, two typical markers of the "conventional" early endosomes. Furthermore, these structures have a neutral pH, whereas early endosomes are acidic. After several hours, SV40 viruses leave caveosomes to enter the ER, using tubular transport intermediates.

Caveosomes also contain CTxB after co-internalization from the plasma membrane, as opposed to markers of clathrin-dependent endocytosis (e.g., Tf) and fluid phase markers (e.g., dextran). They may support the retrograde transport of CTx to the Golgi apparatus (Nichols, 2002; see below). No direct evidence in support of this hypothesis has been obtained, however, and follow-up studies showed that caveosomes and "conventional" EE communicate in an intricate manner (Pelkmans *et al*., 2004). Therefore, it remains possible that CTx can go in and out of caveosomes independently of trafficking to the TGN.

## CONCLUSION

The transport of bacterial and plant toxins in cells is of great interest both in fundamental science and in medicine. First, the understanding of these processes has provided important clues about endocytic mechanisms, such as the early suggestion of the existence of a clathrin-independent pathway (Montesano *et al*., 1982; Moya *et al*., 1985; Sandvig *et al*., 1987) or the retrograde transport of proteins across the Golgi apparatus (Johannes and Goud, 1998; Sandvig *et al*., 1992). The mechanisms that govern these different pathways are still poorly understood. It is highly probable that they carry unique and specific functions in the cell and that toxins have adapted to them so that they can be targeted to the right intracellular compartment. While most of the current knowledge was generated by using pharmacological inhibitors, which often lack specificity, new technologies such as live imaging and RNA interference will undoubtedly advance the molecular understanding of these pathways and the role they play in the pathology of toxins. The role of the different endocytic pathways in intracellular toxin trafficking remains obscure, and so far no clear correlation has been found between sorting at the plasma membrane and the ultimate fate of internalized molecules within the cell (Johannes and Lamaze, 2002).

The intracellular trafficking of toxins has also been used to convert them into targeting devices for therapeutic purposes. Indeed, toxins are specialized in the transport of protein moieties, i.e., their catalytic A-subunits, from the outside of cells into the cytosol. This type of transport is also the central theme of the cellular cross-presentation process observed with professional antigen presenting cells (Ackerman and Cresswell, 2004; Guermonprez and Amigorena, 2005), which leads to the presentation of antigens on MHC class I molecules, CD8+ T-cell priming, and immunity against viruses and cancer. Receptor-binding subunits of toxins are therefore being used to develop therapeutic vaccination strategies (Smith *et al*., 2002; Tartour *et al*., 2002). Recently, these strategies have mostly been based on two toxins that specifically target dendritic cells, the gatekeeper of cellular immunity: *Bordetella pertussis* adenylate cyclase toxin (Fayolle *et al*., 2004; Fayolle *et al*., 1999; Fayolle *et al*., 1996; Saron *et al*., 1997; Schlecht *et al*., 2004) and STxB (Haicheur *et al*., 2003; Haicheur *et al*., 2000; Lee *et al*., 1998; Smith *et al*., 2002). Other therapeutic approaches based on direct cancer cell targeting are currently being developed (Farkas-Himsley *et al*., 1995; Ishitoya *et al*., 2004). In conclusion, protein toxins may pay back some of the death toll that they still cause to humans.

## ACKNOWLEDGMENTS

The work in the authors' laboratory was supported by grants from Ligue Nationale contre le Cancer, Association pour la Recherche sur le Cancer (#5177 and

#3105), Fondation de France, Action Concertée Incitative-Jeunes Chercheurs (#5233), and GIS Prion from Ministère de la Recherche.

## REFERENCES


Abrami, L., Lindsay, M., Parton, R.G., Leppla, S.H. and van der Goot, F.G. (2004). Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. *J. Cell. Biol*. **166**, 645–651.

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and Van Der Goot, F.G. (2003). Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J. Cell. Biol*. **160**, 321–328.

Ackerman, A.L. and Cresswell, P. (2004). Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat. Immunol*. **5**, 678–684.

Amyere, M., Mettlen, M., Van Der Smissen, P., Platek, A., Payrastre, B., Veithen, A. and Courtoy, P. J. (2002). Origin, originality, functions, subversions and molecular signalling of macropinocytosis. *Int. J. Med. Microbiol*. **291**, 487–494.

Arab, S. and Lingwood, C.A. (1998). Intracellular targeting of the endoplasmic reticulum/nuclear envelope by retrograde transport may determine cell hypersensitivity to verotoxin via globotriaosyl ceramide fatty acid isoform traffic. *J. Cell. Physiol*. **177**, 646–660.

Arighi, C.N., Hartnell, L.M., Aguilar, R.C., Haft, C.R. and Bonifacino, J.S. (2004). Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. *J. Cell. Biol*. **165**, 123–133.

Bacia, K., Majoul, I.V. and Schwille, P. (2002). Probing the endocytic pathway in live cells using dual-color fluorescence cross-correlation analysis. *Biophys. J*. **83**, 1184–1193.

Bantel-Schaal, U., Hub, B. and Kartenbeck, J. (2002). Endocytosis of adeno-associated virus type 5 leads to accumulation of virus particles in the Golgi compartment. *J. Virol*. **76**, 2340–2349.

Bastiaens, P.I., Majoul, I.V., Verveer, P.J., Soling, H.D. and Jovin, T. M. (1996). Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *Embo. J*. **15**, 4246–4253.

Beaumelle, B., Alami, M. and Hopkins, C.R. (1993). ATP-dependent translocation of ricin across the membrane of purified endosomes. *J. Biol. Chem*. **268**, 23661–23669.

Beranger, F., Mange, A., Goud, B. and Lehmann, S. (2002). Stimulation of PrPC retrograde transport towards the endoplasmic reticulum increases accumulation of PrPSc in prion-infected cells. *J. Biol. Chem*. **277**, 38972–38977.

Birkeli, K. A., Llorente, A., Torgersen, M.L., Keryer, G., Tasken, K. and Sandvig, K. (2003). Endosome-to-Golgi transport is regulated by protein kinase A type II alpha. *J. Biol. Chem*. **278**, 1991–1997.

Blot, G., Janvier, K., Le Panse, S., Benarous, R. and Berlioz-Torrent, C. (2003). Targeting of the human immunodeficiency virus type 1 envelope to the trans-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity. *J. Virol*. **77**, 6931–6945.

Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, J.A. (2001). Identification of the cellular receptor for anthrax toxin. *Nature* **414**, 225–229.

Brodsky, F.M., Chen, C.Y., Knuehl, C., Towler, M.C. and Wakeham, D. E. (2001). Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell. Dev. Biol*. **17**, 517–568.

Brown, D.A. and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem*. **275**, 17221–17224.

Chen, A., AbuJarour, R.J. and Draper, R.K. (2003). Evidence that the transport of ricin to the cytoplasm is independent of both Rab6A and COPI. *J. Cell. Sci*. **116**, 3503–3510.

Chen, A., Hu, T., Mikoryak, C. and Draper, R.K. (2002a). Retrograde transport of protein toxins under conditions of COPI dysfunction. *Biochim. Biophys. Acta*. **1589**, 124–139.

Chen, J.L., Ahluwalia, J.P. and Stamnes, M. (2002b). Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. *J. Biol. Chem*. **277**, 35682–35687.

Choudhury, A., Dominguez, M., Puri, V., Sharma, D.K., Narita, K., Wheatley, C.L., Marks, D.L. and Pagano, R.E. (2002). Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest*. **109**, 1541–1550.

Cid, D., Blanco, M., Blanco, J.E., Ruiz Santa Quiteira, J.A., de la Fuente, R. and Blanco, J. (1996). Serogroups, toxins, and antibiotic resistance of Escherichia coli strains isolated form diarrhoeic goat kids in Spain. *Vet. Microbiol*. **53**, 349–354.

Collier, R.J. (2001). Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon*. **39**, 1793–1803.

Conner, S.D. and Schmid, S.L. (2003). Differential requirements for AP-2 in clathrin-mediated endocytosis. *J. Cell. Biol*. **162**, 773–779.

Contamin, S., Galmiche, A., Doye, A., Flatau, G., Benmerah, A. and Boquet, P. (2000). The p21 rho-activating toxin cytotoxic necrotizing factor 1 is endocytosed by a clathrin-independent mechanism and enters the cytosol by an acidic-dependent membrane translocation step. *Mol. Biol. Cell*. **11**, 1775–1787.

Cosson, P. and Letourneur, F. (1997). Coatomer (COPI)-coated vesicles: role in intracellular transport and protein sorting. *Curr. Opin. Cell. Biol*. **9**, 484–487.

Crotzer, V.L., Mabardy, A.S., Weiss, A. and Brodsky, F.M. (2004). T cell receptor engagement leads to phosphorylation of clathrin heavy chain during receptor internalization. *J. Exp. Med*. **199**, 981–991.

Crump, C.M., Bruun, B., Bell, S., Pomeranz, L.E., Minson, T. and Browne, H.M. (2004). Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins. *J. Gen. Virol*. **85**, 3517–3527.

Cunningham, K., Lacy, D.B., Mogridge, J. and Collier, R.J. (2002). Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc. Natl. Acad. Sci. U S A* **99**, 7049–7053. Epub 2002 May 7007.

Cupers, P., Veithen, A., Kiss, A., Baudhuin, P. and Courtoy, P. J. (1994). Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. *J. Cell. Biol*. **127**, 725–735.

Damke, H., Baba, T., Warnock, D.E. and Schmid, S.L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell. Biol*. **127**, 915–934.

Damke, H., Gossen, M., Freundlieb, S., Bujard, H. and Schmid, S.L. (1995). Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Methods. Enzymol*. **257**, 209–220.

Dascher, C., Matteson, J., and Balch, W.E. (1994). Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. *J. Biol. Chem*. **269**, 29363–29366.

Diaz, E. and Pfeffer, S.R. (1998). TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell*. **93**, 433–443.

Donta, S.T., Tomicic, T.K. and Donohue-Rolfe, A. (1995). Inhibition of Shiga-like toxins by brefeldin A. *J. Infect. Dis*. **171**, 721–724.

Duran, J.M., Valderrama, F., Castel, S., Magdalena, J., Tomas, M., Hosoya, H., Renau-Piqueras, J., Malhotra, V. and Egea, G. (2003). Myosin motors and not actin comets are mediators of the actin-based Golgi-to-endoplasmic reticulum protein transport. *Mol. Biol. Cell*. **14**, 445–459.

Dykstra, M.L., Cherukuri, A. and Pierce, S.K. (2001). Floating the raft hypothesis for immune receptors: access to rafts controls receptor signaling and trafficking. *Traffic* **2**, 160–166.

Eiklid, K. and Olsnes, S. (1980). Interaction of Shigella shigae cytotoxin with receptors on sensitive and insensitive cells. *J. Rec. Res*. **1**, 199–213.

El-Azami-El-Idrissi, M., Bauche, C., Loucka, J., Osicka, R., Sebo, P., Ladant, D. and Leclerc, C. (2003). Interaction of Bordetella pertussis adenylate cyclase with CD11b/CD18: Role of toxin acylation and identification of the main integrin interaction domain. *J. Biol. Chem*. **278**, 38514–38521. Epub 32003 Jul 38528.

Fabbri, A., Gauthier, M. and Boquet, P. (1999). The 5′ region of cnf1 harbors a translational regulatory mechanism for CNF1 synthesis and encodes the cell-binding domain of the toxin. *Mol. Microbiol*. **33**, 108–118.

Falbo, V., Pace, T., Picci, L., Pizzi, E. and Caprioli, A. (1993). Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of Escherichia coli. *Infect. Immun*. **61**, 4909–4914.

Falguières, T., Mallard, F., Baron, C.L., Hanau, D., Lingwood, C., Goud, B., Salamero, J. and Johannes, L. (2001). Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent resistant membranes. *Mol. Biol. Cell*. **12**, 2453–2468.

Farkas-Himsley, H., Hill, R., Rosen, B., Arab, S. and Lingwood, C.A. (1995). The bacterial colicin active against tumor cells *in vitro* and *in vivo* is verotoxin 1. *Proc. Natl. Acad. Sci. USA* **92**, 6996–7000.

Fayolle, C., Bauche, C., Ladant, D. and Leclerc, C. (2004). Bordetella pertussis adenylate cyclase delivers chemically coupled CD8(+) T-cell epitopes to dendritic cells and elicits CTL *in vivo. Vaccine* **23**, 604–614.

Fayolle, C., Ladant, D., Karimova, G., Ullmann, A. and Leclerc, C. (1999). Therapy of murine tumors with recombinant Bordetella pertussis adenylate cyclase carrying a cytotoxic T cell epitope. *J. Immunol*. **162**, 4157–4162.

Fayolle, C., Sebo, P., Ladant, D., Ullmann, A. and Leclerc, C. (1996). *In vivo* induction of CTL responses by recombinant adenylate cyclase of Bordetella pertussis carrying viral CD8+ T cell epitopes. *J. Immunol*. **156**, 4697–4706.

Folsch, H., Pypaert, M., Schu, P. and Mellman, I. (2001). Distribution and function of AP-1 clathrin adaptor complexes in polarized epithelial cells. *J. Cell. Biol*. **152**, 595–606.

Fra, A.M., Williamson, E., Simons, K. and Parton, R.G. (1994). Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem*. **269**, 30745–30748.

Fujinaga, Y., Wolf, A.A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M.G., Rapoport, T., Holmes, R.K. and Lencer, W.I. (2003). Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to the endoplasmic reticulum. *Mol. Biol. Cell*. **14**, 4783–4793.

Ghosh, P., Dahms, N.M. and Kornfeld, S. (2003). Mannose 6-phosphate receptors: new twists in the tale. *Nat. Rev. Mol. Cell. Biol*. **4**, 202–212.

Ghosh, R.N., Mallet, W.G., Soe, T.T., McGraw, T.E. and Maxfield, F. R. (1998). An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. *J. Cell. Biol*. **142**, 923–936.

Gilbert, A., Paccaud, J.P., Foti, M., Porcheron, G., Balz, J. and Carpentier, J.L. (1999). Direct demonstration of the endocytic function of caveolae by a cell-free assay. *J. Cell. Sci*. **112**, 1101–1110.

Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T. and Pepperkok, R. (1999). Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nature Cell. Biol*. **1**, 423–430.

Grimmer, S., Iversen, T.G., van Deurs, B. and Sandvig, K. (2000). Endosome to golgi transport of ricin is regulated by cholesterol. *Mol. Biol. Cell*. **11**, 4205–4216.

Gruenberg, J. and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. *Nat. Rev. Mol. Cell. Biol*. **5**, 317–323.

Guermonprez, P. and Amigorena, S. (2005). Pathways for antigen cross presentation. *Springer Semin. Immunopathol*. **26**, 257–271.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of Bordetella pertussis binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *J. Exp. Med*. **193**, 1035–1044.

Haicheur, N., Benchetrit, F., Amessou, M., Leclerc, C., Falguières, T., Fayolle, C., Bismuth, E., Fridman, W.H., Johannes*, L. and Tartour*, E. (2003). The B-subunit of Shiga toxin coupled to full-size protein elicits humoral and cellular immune responses associated with a TH1 dominant polarization. *Int. Immunol*. **15**, 1161–1171 (*principal investigators).

Haicheur, N., Bismuth, E., Bosset, S., Adotevi, O., Warnier, G., Lacabanne, V., Regnault, A., Desaymard, C., Amigorena, S., Riccardi-Castagnoli, P. *et al*. (2000). The B-subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I restricted presentation of peptides derived from exogenous antigens. *J. Immunol*. **165**, 3301–3308 (*principal investigators).

Harder, T., Scheiffele, P., Verkade, P. and Simons, K. (1998). Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell. Biol*. **141**, 929–942.

Hay, J.C., Klumperman, J., Oorschot, V., Steegmaier, M., Kuo, C.S. and Scheller, R. H. (1998). Localization, dynamics, and protein interactions reveal distinct roles for ER and Golgi SNAREs. *J. Cell. Biol*. **141**, 1489–1502.

Henley, J.R., Krueger, E.W., Oswald, B.J. and McNiven, M.A. (1998). Dynamin-mediated internalization of caveolae. *J. Cell. Biol*. **141**, 85–99.

Ishitoya, S., Kurazono, H., Nishiyama, H., Nakamura, E., Kamoto, T., Habuchi, T., Terai, A., Ogawa, O. and Yamamoto, S. (2004). Verotoxin induces rapid elimination of human renal tumor xenografts in SCID mice. *J. Urol*. **171**, 1309–1313.

Iversen, T.G., Skretting, G., Llorente, A., Nicoziani, P., van Deurs, B. and Sandvig, K. (2001). Endosome to golgi transport of ricin is independent of clathrin and of the Rab9 and Rab11 GTPases. *Mol. Biol. Cell*. **12**, 2099–2107.

Jackson, M.E., Simpson, J. C., Girod, A., Pepperkok, R., Roberts, L.M. and Lord, J.M. (1999). The KDEL retrieval system is exploited by pseudomonas exotoxin A, but not by shiga-like toxin-1, during retrograde transport from the golgi complex to the endoplasmic reticulum. *J. Cell. Sci*. **112**, 467–475.

Johannes, L. (2002). The Shiga toxin B-subunit system: Retrograde transport, intracellular vectorization, and more. *Am. J. Physiol. Gastrointest. Liver. Physiol*. **283**, G1–G7.

Johannes, L. and Goud, B. (1998). Surfing on a retrograde wave: how does Shiga toxin reach the endoplasmic reticulum? *Trends. Cell. Biol*. **8**, 158–162.

Johannes, L. and Lamaze, C. (2002). Clatrin-dependent or not: Is it still the question? *Traffic* **3**, 443–451.

Johannes, L., Tenza, D., Antony, C. and Goud, B. (1997). Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. *J. Biol. Chem*. **272**, 19554–19561.

Katagiri, Y.U., Mori, T., Nakajima, H., Katagiri, C., Taguchi, T., Takeda, T., Kiyokawa, N. and Fujimoto, J. (1999). Activation of src family kinase yes induced by shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. *J. Biol. Chem*. **274**, 35278–35282.

Kim, J.H., Johannes, L., Goud, B., Antony, C., Lingwood, C.A., Daneman, R. and Grinstein, S. (1998). Noninvasive measurement of the pH of the endoplasmic reticulum at rest and during calcium release. *Proc. Natl. Acad. Sci. USA* **95,** 2997–3002.

Kirkham, M., Fujita, A., Chadda, R., Nixon, S.J., Kurzchalia, T.V., Sharma, D.K., Pagano, R.E., Hancock, J.F., Mayor, S. and Parton, R.G. (2005). Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. *J. Cell. Biol*. **168**, 465–476.

Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R. G. and Gruenberg, J. (1998). A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature* **392,** 193–197.

Kounnas, M.Z., Morris, R.E., Thompson, M.R., FitzGerald, D.J., Strickland, D.K. and Saelinger, C.B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem*. **267,** 12420–12423.

Kovbasnjuk, O., Edidin, M. and Donowitz, M. (2001). Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J. Cell. Sci*. **114,** 4025–4031.

Kurzchalia, T. (2003). Anthrax toxin rafts into cells. *J. Cell. Biol*. **160,** 295–296.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C.G., Benmerah, A. and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell*. **7,** 661–671.

Lauvrak, S.U., Llorente, A., Iversen, T.G. and Sandvig, K. (2002). Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. *J. Cell. Sci*. **115,** 3449–3456.

Lauvrak, S.U., Torgersen, M.L. and Sandvig, K. (2004). Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. *J. Cell. Sci*. **117,** 2321–2331.

Le PU, Guay, G., Altschuler, Y. and Nabi, I.R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem*. **277,** 3371–3379.

Lee, R.-S., Tartour, E., van der Bruggen, P., Vantomme, V., Goud, B., Fridman, W.-H. and Johannes, L. (1998). Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga toxin. *Eur. J. Immunol*. **28,** 2726–2737.

Lemichez, E., Bomsel, M., Devilliers, G., vanderSpek, J., Murphy, J.R., Lukianov, E.V., Olsnes, S. and Boquet, P. (1997). Membrane translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. *Mol. Microbiol*. **23,** 445–457.

Lencer, W.I., Constable, C., Moe, S., Jobling, M.G., Webb, H.M., Ruston, S., Madara, J.L., Hirst, T.R. and Holmes, R.K. (1995). Targeting of cholera toxin and Escherichia coli heat-labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell. Biol*. **131,** 951–962.

Leppla, S.H. (1988). Production and purification of anthrax toxin. *Methods. Enzymol*. **165,** 103–116.

Lin, S.X., Gundersen, G.G. and Maxfield, F.R. (2002). Export from pericentriolar endocytic recycling compartment to cell surface depends on stable, detyrosinated (glu) microtubules and kinesin. *Mol. Biol. Cell*. **13,** 96–109.

Llorente, A., Rapak, A., Schmid, S.L., van Deurs, B. and Sandvig, K. (1998). Expression of mutant dynamin inhibits toxicity and transport of endocytosed ricin to the Golgi apparatus. *J. Cell. Biol*. **140,** 553–563.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M. and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. *Embo. J*. **12,** 677–682.

Lord, J.M. and Roberts, L.M. (1998). Retrograde transport: going against the flow. *Curr. Biol*. **8,** R56–R58.

Lu, L., Tai, G. and Hong, W. (2004). Autoantigen Golgin-97, an effector of Arl1 GTPase, participates in traffic from the endosome to the trans-golgi network. *Mol. Biol. Cell*. **15,** 4426–4443.

Luna, A., Matas, O.B., Martinez-Menarguez, J.A., Mato, E., Duran, J.M., Ballesta, J., Way, M. and Egea, G. (2002). Regulation of protein transport from the Golgi complex to the endoplasmic reticulum by CDC42 and N-WASP. *Mol. Biol. Cell*. **13,** 866–879.

Ma, J. and Lindquist, S. (2001). Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. *Proc. Natl. Acad. Sci. USA* **98,** 14955–14960.

Madshus, I. H., Stenmark, H., Sandvig, K. and Olsnes, S. (1991). Entry of diphtheria toxin-protein A chimeras into cells. *J. Biol. Chem*. **266,** 17446–17453.

Majoul, I., Sohn, K., Wieland, F.T., Pepperkok, R., Pizza, M., Hillemann, J. and Soling, H.D. (1998). KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. *J. Cell. Biol*. **143,** 601–612.

Majoul, I.V., Bastiaens, P.I. and Soling, H.D. (1996). Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J. Cell. Biol*. **133,** 777–789.

Mallard, F. and Johannes, L. (2002). Shiga toxin B-subunit as a tool to study retrograde transport. In: *Methods Mol Med Shiga Toxin Methods and Protocols* (Edited by: D Philpott and F Ebel), Vol. **73,** Chapter 17, pp. 209–220. Humana Press; Totowa, New Jersey.

Mallard, F., Tang, B.L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W.J., Goud, B. and Johannes, L. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *J. Cell. Biol*. **156,** 653–664.

Mallard, F., Tenza, D., Antony, C., Salamero, J., Goud, B. and Johannes, L. (1998). Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin B-fragment transport. *J. Cell. Biol*. **143,** 973–990.

Massol, R.H., Larsen, J.E., Fujinaga, Y., Lencer, W.I. and Kirchhausen, T. (2004). Cholera toxin toxicity does not require functional Arf6- and dynamin-dependent endocytic pathways. *Mol. Biol. Cell*. **15,** 3631–3641. Epub 2004 May 3614.

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., Blanc, N.S., Matile, S., Dubochet, J., Sadoul, R., *et al*. (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science* **303,** 531–534.

McIntosh, D., Timar, J. and Davies, A.J. (1990). The intracellular movement and cycling of ricin. *Eur. J. Cell. Biol*. **52,** 77–86.

Meyer, C., Zizioli, D., Lausmann, S., Eskelinen, E.L., Hamann, J., Saftig, P., von Figura, K. and Schu, P. (2000). $\mu$1A-adaptin-deficient mice: lethality, loss of AP-1 binding, and rerouting of mannose 6-phosphate receptors. *Embo. J*. **19,** 2193–2203.

Miesenbock, G. and Rothman, J.E. (1995). The capacity to retrieve escaped ER proteins extends to the trans-most cisterna of the Golgi stack. *J. Cell. Biol*. **129,** 309–319.

Milne, J.C., Furlong, D., Hanna, P.C., Wall, J.S. and Collier, R.J. (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem*. **269,** 20607–20612.

Minshall, R., Tiruppathi, C., Vogel, S., Niles, W., Gilchrist, A., Hamm, H. and Malik, A. (2000). Endothelial cell-surface gp60 activates vesicle formation and trafficking via G(i)-coupled Src kinase signaling pathway. *J. Cell. Biol*. **150,** 1057–1070.

Mogridge, J., Mourez, M. and Collier, R.J. (2001). Involvement of domain 3 in oligomerization by the protective antigen moiety of anthrax toxin. *J. Bacteriol*. **183,** 2111–2116.

Montesano, R., Roth, J., Robert, A. and Orci, L. (1982). Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature* **296,** 651–653.

Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. and Boquet, P. (1985). Inhibition of coated pit formation in HEP2 cells blocks the cytotoxicity of diphteria toxin but not that of ricin toxin. *J. Cell. Biol*. **101,** 548–559.

Mukherjee, S., Ghosh, R.N. and Maxfield, F.R. (1997). Endocytosis. *Physiol. Rev*. **77,** 759–803.

Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* **115,** 377–388.

Nabi, I.R. and Le, P.U. (2003). Caveolae/raft-dependent endocytosis. *J. Cell. Biol*. **161,** 673–677.

Nambiar, M.P., Oda, T., Chen, C., Kuwazuru, Y. and Wu, H. C. (1993). Involvement of the Golgi region in the intracellular trafficking of cholera toxin. *J. Cell. Physiol*. **154,** 222–228.

Natarajan, R. and Linstedt, A.D. (2004). A cycling cis-Golgi protein mediates endosome-to-Golgi traffic. *Mol. Biol. Cell*. **15,** 4798–4806.

Nichols, B.J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell. Biol*. **15,** 15.

Nichols, B.J. (2003). GM1-containing lipid rafts are depleted within clathrin-coated pits. *Curr. Biol*. **13,** 686–690.

Nichols, B.J., Kenworthy, A.K., Polishchuk, R. S., Lodge, R., Roberts, T.H., Hirschberg, K., Phair, R.D. and Lippincott-Schwartz, J. (2001). Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell. Biol*. **153,** 529–541.

Oh, P., McIntosh, D.P. and Schnitzer, J.E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell. Biol*. **141,** 101–114.

Orlandi, P.A. and Fishman, P.H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell. Biol*. **141,** 905–915.

Owen, D.J., Collins, B.M. and Evans, P.R. (2004). Adaptors for clathrin coats: structure and function. *Annu. Rev. Cell. Dev. Biol*. **20,** 153–191.

Palade, G.E. (1953). The fine structure of blood capillaries. *J. Appl. Phys*. **24,** 1424.

Pang, H., Le, P.U. and Nabi, I.R. (2004). Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of cholera toxin to the Golgi apparatus. *J. Cell. Sci*. **117,** 1421–1430.

Papini, E., Rappuoli, R., Murgia, M. and Montecucco, C. (1993). Cell penetration of diphtheria toxin. Reduction of the interchain disulfide bridge is the rate-limiting step of translocation in the cytosol. *J. Biol. Chem*. **268,** 1567–1574.

Parton, R.G. (1994). Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J. Histochem. Cytochem*. **42,** 155–166.

Parton, R.G. and Richards, A.A. (2003). Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. *Traffic* **4,** 724–738.

Pelham, H.R. (1996). The dynamic organization of the secretory pathway. *Cell. Struct. Funct*. **21,** 413–419.

Pelkmans, L., Burli, T., Zerial, M. and Helenius, A. (2004). Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell*. **118,** 767–780.

Pelkmans, L. and Helenius, A. (2002). Endocytosis via caveolae. *Traffic* **3,** 311–320.

Pelkmans, L., Kartenbeck, J. and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell. Biol*. **3,** 473–483.

Pelkmans, L., Puntener, D. and Helenius, A. (2002). Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* **296,** 535–539.

Pernet-Gallay, K., Antony, C., Johannes, L., Goud, B., Bornens, M. and Rios, R.M. (2002). The overexpression of GMAP210 blocks anterograde and retrograde transport between the ER and the Golgi apparatus. *Traffic* **3,** 822–832.

Petosa, C., Collier, R. J., Klimpel, K.R., Leppla, S.H. and Liddington, R. C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Puri, V., Watanabe, R., Singh, R.D., Dominguez, M., Brown, J.C., Wheatley, C.L., Marks, D.L. and Pagano, R.E. (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell. Biol*. **154,** 535–547.

Ramegowda, B. and Tesh, V.L. (1996). Differentiation-associated toxin receptor modulation, cytokine production, and sensitivity to Shiga-like toxins in human monocytes and monocytic cell lines. *Infect. Immun*. **64,** 1173–1180.

Rappoport, J.Z., Simon, S.M. and Benmerah, A. (2004). Understanding living clathrin-coated pits. *Traffic* **5,** 327–337.

Rodal, S.K., Skretting, G., Garred, O., Vilhardt, F., van Deurs, B. and Sandvig, K. (1999). Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell*. **10,** 961–974.

Rogel, A. and Hanski, E. (1992). Distinct steps in the penetration of adenylate cyclase toxin of Bordetella pertussis into sheep erythrocytes. Translocation of the toxin across the membrane. *J. Biol. Chem*. **267,** 22599–22605.

Roth, T.F. and Porter, K.R. (1964). Yolk protein uptake in the oocyte of the mosquito aedes aegypti. L.*J. Cell. Biol*. **20**, 313–332.

Sabharanjak, S., Sharma, P., Parton, R.G. and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell*. **2**, 411–423.

Saint-Pol, A., Yélamos, B., Amessou, M., Mills, I., Dugast, M., Tenza, D., Schu, P., Antony, C., McMahon, H.T., Lamaze, C. and Johannes, L. (2004). Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Dev. Cell*. **6**, 525–538.

Sandvig, K., Garred, O., Prydz, K., Kozlov, J.V., Hansen, S.H. and van Deurs, B. (1992). Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* **358**, 510–512.

Sandvig, K., Garred, O., van Helvoort, A., van Meer, G. and van Deurs, B. (1996). Importance of glycolipid synthesis for butyric acid-induced sensitization to shiga toxin and intracellular sorting of toxin in A431 cells. *Mol. Biol. Cell*. **7**, 1391–1404.

Sandvig, K., Olsnes, S., Brown, J.E., Petersen, O.W. and van Deurs, B. (1989). Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from Shigella dysenteriae 1. *J. Cell. Biol*. **108**, 1331–1343.

Sandvig, K., Olsnes, S., Petersen, O.W. and van Deurs, B. (1987). Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell. Biol*. **105**, 679–689.

Sandvig, K., Spilsberg, B., Lauvrak, S.U., Torgersen, M.L., Iversen, T.G. and van Deurs, B. (2004). Pathways followed by protein toxins into cells. *Int. J. Med. Microbiol*. **293**, 483–490.

Saron, M.F., Fayolle, C., Sebo, P., Ladant, D., Ullmann, A. and Leclerc, C. (1997). Anti-viral protection conferred by recombinant adenylate cyclase toxins from Bordetella pertussis carrying a CD8+ T cell epitope from lymphocytic choriomeningitis virus. *Proc. Natl. Acad. Sci. USA* **94**, 3314–3319.

Schapiro, F.B., Lingwood, C., Furuya, W. and Grinstein, S. (1998). pH-independent retrograde targeting of glycolipids to the Golgi complex. *Am. J. Physiol*. **274**, C319–C332.

Schlecht, G., Loucka, J., Najar, H., Sebo, P. and Leclerc, C. (2004). Antigen targeting to CD11b allows efficient presentation of CD4+ and CD8+ T cell epitopes and *in vivo* Th1-polarized T cell priming. *J. Immunol*. **173**, 6089–6097.

Seaman, M.N. (2004). Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. *J. Cell. Biol*. **165**, 111–122.

Seaman, M.N., McCaffery, J. M. and Emr, S. D. (1998). A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell. Biol*. **142**, 665–681.

Sharma, D.K., Brown, J.C., Choudhury, A., Peterson, T.E., Holicky, E., Marks, D.L., Simari, R., Parton, R.G. and Pagano, R.E. (2004a). Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol. Biol. Cell*. **15**, 3114–3122. Epub 2004 Apr 3123.

Sharma, P., Sabharanjak, S. and Mayor, S. (2002). Endocytosis of lipid rafts: an identity crisis. *Semin. Cell. Dev. Biol*. **13**, 205–214.

Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M. and Mayor, S. (2004b). Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577–589.

Shewan, A.M., van Dam, E.M., Martin, S., Luen, T.B., Hong, W., Bryant, N.J. and James, D.E. (2003). GLUT4 recycles via a trans-Golgi network (TGN) subdomain enriched in Syntaxins 6 and 16 but not TGN38: involvement of an acidic targeting motif. *Mol. Biol. Cell*. **14**, 973–986.

Shogomori, H. and Futerman, A.H. (2001). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. *J. Biol. Chem*. **276**, 9182–9188.

Simons, K. and Ikonen, E. (1997). Functional rafts in cell membranes. *Nature* **387**, 569–572.

Simpson, J.C., Dascher, C., Roberts, L.M., Lord, J.M. and Balch, W.E. (1995). Ricin cytotoxicity is sensitive to recycling between the endoplasmic reticulum and the Golgi complex. *J. Biol. Chem*. **270**, 20078–20083.

Simpson, J.C., Roberts, L.M., Romisch, K., Davey, J., Wolf, D.H. and Lord, J.M. (1999). Ricin A chain utilizes the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS. Lett*. **459**, 80–84.

Simpson, J.C., Smith, D.C., Roberts, L.M. and Lord, J.M. (1998). Expression of mutant dynamin protects cells against diphtheria toxin but not against ricin. *Exp. Cell. Res*. **239**, 293–300.

Singh, R.D., Puri, V., Valiyaveettil, J.T., Marks, D.L., Bittman, R. and Pagano, R.E. (2003). Selective caveolin-1-dependent endocytosis of glycosphingolipids. *Mol. Biol. Cell* **14**, 3254–3265.

Singh, Y., Klimpel, K.R., Arora, N., Sharma, M. and Leppla, S.H. (1994). The chymotrypsin-sensitive site, FFD315, in anthrax toxin protective antigen is required for translocation of lethal factor. *J. Biol. Chem*. **269**, 29039–29046.

Slepnev, V.I. and De Camilli, P. (2000). Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat. Rev. Neurosci*. **1**, 161–172.

Smith, D.C., Lord, J.M., Roberts, L.M., Tartour, E. and Johannes, L. (2002). 1st class ticket to class I: Protein toxin as pathfinders for antigen presentation. *Traffic* **3**, 697–704.

Spiegel, S., Kassis, S., Wilchek, M. and Fishman, P.H. (1984). Direct visualization of redistribution and capping of fluorescent gangliosides on lymphocytes. *J. Cell Biol*. **99**, 1575–1581.

Spilsberg, B., Van Meer, G. and Sandvig, K. (2003). Role of lipids in the retrograde pathway of ricin intoxication. *Traffic* **4**, 544–552.

Steele-Mortimer, O., Knodler, L.A. and Finlay, B.B. (2000). Poisons, ruffles, and rockets: bacterial pathogens and the host cell cytoskeleton. *Traffic* **1**, 107–118.

Stoddart, A., Dykstra, M.L., Brown, B.K., Song, W., Pierce, S.K. and Brodsky, F. M. (2002). Lipid rafts unite signaling cascades with clathrin to regulate BCR internalization. *Immunity* **17**, 451–462.

Stoorvogel, W., Oorschot, V. and Geuze, H.J. (1996). A novel class of clathrin-coated vesicles budding from endosomes. *J. Cell Biol*. **132**, 21–33.

Storrie, B., Pepperkok, R. and Nilsson, T. (2000). Breaking the COPI monopoly on Golgi recycling. *Trends Cell Biol*. **10**, 385–391.

Tai, G., Lu, L., Wang, T.L., Tang, B.L., Goud, B., Johannes, L. and Hong, W. (2004). Participation of syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the TGN. *Mol. Biol. Cell* **15**, 4011–4022.

Tartour, E., Benchetrit, F., Haicheur, N., Adotevi, O. and Fridman, W. H. (2002). Synthetic and natural non-live vectors: rationale for their clinical development in cancer vaccine protocols. *Vaccine* **20**, A32–A39.

Tesh, V.L., Ramegowda, B. and Samuel, J.E. (1994). Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. *Infect. Immun*. **62**, 5085–5094.

Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol. Biol. Cell* **13**, 238–250.

Torgersen, M.L., Skretting, G., van Deurs, B. and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci*. **114**, 3737–3747.

Tran, D., Carpentier, J.-L., Sawano, F., Gorden, P. and Orci, L. (1987). Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc. Natl. Acad. Sci. USA* **84**, 7957–7961.

Tsai, B., Rodighiero, C., Lencer, W.I. and Rapoport, T. (2001). Protein disulfide isomerase acts as a redox-dependent chaperone to unfold Cholera toxin. *Cell* **104**, 937–948.

Tsai, B., Ye, Y. and Rapoport, T.A. (2002). Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat. Rev. Mol. Cell. Biol*. **3**, 246–255.

Valderrama, F., Duran, J. M., Babia, T., Barth, H., Renau-Piqueras, J. and Egea, G. (2001). Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. *Traffic* **2**, 717–726.

Valdivia, R. H., Baggott, D., Chuang, J.S. and Schekman, R.W. (2002). The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins. *Dev. Cell*. **2**, 283–294.

van Deurs, B., Sandvig, K., Petersen, O.W., Olsnes, S., Simons, K. and Griffiths, G. (1988). Estimation of the amount of internalized ricin that reaches the trans-Golgi network. *J. Cell. Biol*. **106**, 253–267.

van Setten, P.A., Monnens, L.A., Verstraten, R.G., van den Heuvel, L. P. and van Hinsbergh, V. W. (1996). Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. *Blood* **88**, 174–183.

Wesche, J., Rapak, A. and Olsnes, S. (1999). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J. Biol. Chem*. **274**, 34443–34449.

White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Echard, A., Goud, B. and Stelzer, E. H. K. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell. Biol*. **147**, 743–759. (*co-first author)

Wilcke, M., Johannes, L., Galli, T., Mayau, V., Goud, B. and Salamero, J. (2000). Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-Golgi network. *J. Cell Biol*. **151**, 1207–1220.

Wolf, A.A., Fujinaga, Y. and Lencer, W.I. (2002). Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem*. **277**, 16249–16256.

Wolf, A.A., Jobling, M.G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J.L., Holmes, R.K. and Lencer, W.I. (1998). Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell. Biol*. **141**, 917–927.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15**, 245–250.

Yoshida, T., Chen, C.C., Zhang, M.S. and Wu, H.C. (1991). Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and Pseudomonas toxin. *Exp. Cell Res*. **192**, 389–395.

CHAPTER

# 9

# Bacterial toxins and virulence factors targeting the actin cytoskeleton and intercellular junctions

*Michel R. Popoff and Bradley G. Stiles*

## INTRODUCTION

Actin is a highly conserved, versatile protein of ~43 kDa (monomeric form) found in eucaryotes throughout the biosphere, and it represents one of the most abundant proteins in all cells of multi-cellular organisms. In addition to the actin-like molecules found in plants and yeast, an ancient homologue of actin called *MreB* also exists in procaryotes such as non-spherical bacteria (Löwe *et al*., 2004). Although the role of MreB is not clearly defined, the molecule does share a remarkably similar conformation with eucaryotic actin. The filamentous forms of MreB may provide at least three functions for a procaryote that include: (i) a cytoskeleton-like structure that affords a distinct shape; (ii) segregation of plasmid as well as chromosomal DNA during mitosis; and (iii) cell polarity.

One focused aspect of this chapter, as well as the target of various bacterial toxins and secreted effector molecules, is eucaryotic actin, which assembles into organized filaments regulated by a variety of cytoskeletal proteins. Actin filaments can form a dynamic meshwork within a cell that extends throughout the cytosol (stress fibers) and underneath the plasma membrane (cortical actin), thus representing the main structure responsible for eucaryotic cell morphology. The actin-based cytoskeleton also controls many cellular functions critical for eucaryotic life including locomotion, endocytosis, exocytosis, trafficking of intracellular organelles, as well as maintenance of intercellular connections known as tight (TJs) and adherens (AJs) junctions. Since extracellular junctions are essential in building cell barriers, like the epithelia and endothelia that protect an organism from the environment as well as delineate internal compartments, pathogen manipulation of host cells via the actin cytoskeleton provides an effective means to breach a host's containment capabilities and gain access to essential nutrients from more appropriate niches throughout the body. Additionally, diversion of cytoskeletal processes provides a means for pathogens to avoid phagocytosis by macrophages. This "avoidance behavior" employed by a microbial invader circumvents a primary mechanism used by the host's immune system for clearing unwanted entities from the body.

One way that bacterial pathogens can obtain nutrients from a host and avoid the immune system is through potent protein toxins secreted into the environment. Certain Gram-positive and Gram-negative bacteria have evolved this method of manipulating the host via cell intoxication. Structurally, many bacterial toxins typically contain an "A" (enzymatic) and "B" (cell binding/translocation) domain located on one or more protein molecules. The "A-B" toxins act at a distance from the bacterium, and after binding to specific cell-surface receptors followed by internalization, these proteins can cause irreversibly profound cellular changes via various mechanisms. As described below, a large number of these bacterial toxins can modify the actin cytoskeleton by either direct interactions with

actin monomers or via altering Rho-GTPases, which then subsequently regulate actin polymerization. The main effects of these toxins upon a host include destabilization of epithelial and/or endothelial cell barriers, which leads to increased permeability and extensive tissue damage.

In addition to protein toxins secreted into the environment, certain Gram-negative pathogens have also evolved type-III secretion systems that require intimate contact with a target cell. The Gram-negative genera (i.e., *Erwinia*, *Escherichia*, *Pseudomonas*, *Ralstonia*, *Salmonella*, *Shigella*, *Vibrio*, *Yersinia*, and *Xanthomonas*) that possess type-III secretion systems are rather diverse and cause a myriad of diseases in plants as well as animals. Upon pathogen-cell contact that may be facilitated by lipid rafts, virulence factors are "injected" into the cytosol of a targeted cell, which then modulate various functions often involving the actin cytoskeleton.

Structurally, type-III secretion systems are composed of multi-protein complexes that form a "ringed-needle" protrusion called an *injectisome*. The transmembrane segment of this mechanism consists of a basal body similar to that anchoring flagella into the bacterial membrane. In fact, there are common proteins (based upon sequence analysis) found in the flagellar and type-III secretion systems, which suggests a common evolutionary link. The type-III secreted effector molecules generally possess two attributes for proper routing, which include: (i) an amphipathic, non-consensus sequence within the first 20 N-terminal residues; and (ii) a binding site for cytoplasmic chaperone molecules located within the first 140 N-terminal residues (Cornelis, 2002; Lee and Galan, 2004). Some invasive bacteria can translocate type-III secreted proteins that transiently reorganize the actin cytoskeleton, thus facilitating pathogen uptake into normally non-phagocytic cells. Once inside a targeted cell, invasive bacteria can sometimes further exploit the actin cytoskeleton for intra-/inter-cellular movement. Thus, these pathogens enter epithelial cells where they replicate away from the immune surveillance system and subsequently gain access to adjacent tissues, the bloodstream, and finally distal sites throughout a host. In other cases, bacteria use type-III effectors to disrupt the actin cytoskeleton of macrophages and avoid phagocytosis.

The following sections of this chapter delve into the mode of action and cellular effects elicited by various bacterial toxins, as well as type-III effectors, that reorganize the eucaryotic actin cytoskeleton (Figure 9.1). These processes employed by numerous pathogens ultimately play a major role in microbial survival, dissemination, and disease. It is evident that a better understanding of how pathogens interact with, and then exploit, the actin infrastructure of eucaryotes can provide promising means of abrogating, or even preventing, certain diseases in the future. For additional reading on this topic, several excellent reviews summarize quite nicely the manipulations of cellular functions by bacteria via toxins and other virulence factors (Herreros *et al*., 2001; Stebbins and Galan, 2001; Barbieri *et al*., 2002; Doye *et al*., 2002).

## ACTIN CYTOSKELETON AND REGULATION OF ACTIN POLYMERIZATION

The eucaryotic cytoskeleton is built upon three distinct, intercommunicating networks known as microfilaments (6 nm diameter), microtubules (23 nm diameter), and intermediate filaments (10 nm diameter) that are respectively composed of unique proteins like actin, tubulin, and various divergently evolved molecules such as keratin (acidic/basic), lamin, etc. Each filament/tubule type is the result of monomer assembly that constitutes a highly dynamic structure continuously assembled and disassembled in response to extracellular stimuli. The protein monomers and resultant filaments bind a large variety of other proteins, which enable the same filamentous strand to participate in unique functions throughout the cell.

Actin filaments can form stable, diverse structures such as the core of microvilli and contractile apparatus found in muscle cells. In non-muscle cells, the labile actin cytoskeleton forms a dense web underlying the plasma membrane (cortical actin) and is also found as long filaments called *stress fibers* that extend throughout the cytosol. Distinct subcompartments of cortical actin can be distinguished that correspond to different peripheral structures such as filopodia, focal adhesions, lamellipodia, microspikes, and ruffles. In most cells, the actin cytoskeleton is the main determinant of shape and participates in various functions. For example, in epithelial and endothelial cells, the actin structure helps maintain strong intercellular connections and adhesion to the underlying extracellular matrix, while macrophages and neutrophils use the actin-based network for movement, spreading, and phagocytosis (Pollard and Borisy, 2003). The actin cytoskeleton is implicated in many other processes such as cell division (cytokinesis), endocytosis, exocytosis, intracellular transport, membrane organization, and signal transduction. Clearly, actin is involved in many processes critical for eucaryotic life.

In non-muscle cells, actin is in a dynamic equilibrium between monomeric (G-actin) and polymeric or

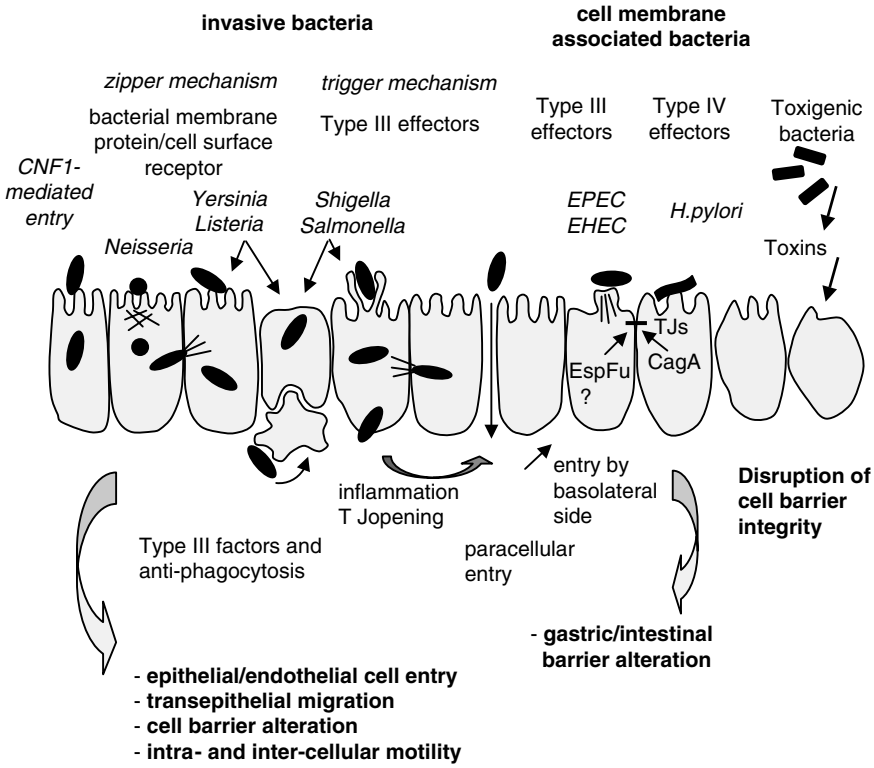



**FIGURE 9.1** Effects of toxins and type-III/IV secreted effectors from various bacteria upon the actin cytoskeleton and epithelial/endothelial cell barriers.

filamentous (F-actin) forms (Pollard *et al*., 2000), which involves a process regulated by various proteins such as profilin, ADF/cofilin, twinfilin, WASP/WAVE, Srv2/CAP, and verprolin/WIP (Figure 9.2). Structurally, actin monomers possess a globular structure with a central groove that binds tightly to an ATP molecule. The side chain of Ser14, and a "sensor loop" consisting of residues Pro70–Asn78 both play an important role in nucleotide binding and subsequent conformation of G-actin linked to either ATP or ADP. G-actin-ATP binds to the growing end of F-actin during polymerization, and ATP is hydrolyzed to ADP, which remains firmly trapped in the actin filament. Depolymerization of F-actin releases G-actin-ADP, which is not readily phosphorylated. Spontaneous dissociation of ADP from G-actin is very slow, but this process is markedly accelerated by the binding of actin monomers to profilin, another multifunctional protein found in all eucaryotes.

Actin filaments are polarized, preferentially polymerizing at the barbed or plus end, which then depolymerizes at the pointed or minus end. The polymerization rate is up to 10 times more evident at the barbed, versus pointed, end. Actin depolymerizing factor (ADF)/cofilin preferentially bind to ADP-bound forms of G-/F-actin (Paavilainen *et al*., 2004), thus increasing by twenty-five-fold the depolymerization rate at the pointed end via twisting of F-actin into a less thermodynamically stable form. This ultimately results in increased concentrations of G-actin-ADP, and then G-actin-ATP, that reach critical levels favorable for actin polymerization (Carlier, 1998; Cooper and Schafer, 2000).

The dynamics of actin monomers/filaments involve four principal stages that include: nucleation, elongation, monomer-polymer equilibrium, and treadmilling. Initiation of actin polymerization takes place by one of three independent mechanisms that include *de novo* nucleation of actin filaments by the actin-related protein (Arp2/3) complex or formins, uncapping of barbed filament ends, and severing of existing filaments to create new barbed ends (Higgs and Pollard, 2001; Suetsugu *et al*., 2002). At least three G-actin-ATP molecules must first associate with Arp2/3 to form a polymerization nucleus sufficient for subsequent F-actin assembly. This process is mainly governed by activated Cdc42 or Rac through WASP or WAVE proteins, respectively (Figure 9.2). From the nucleus, elongation takes place by addition of G-actin-ATP monomers to the barbed end of a nascent filament. Rapid growth at the barbed ends requires high concentrations (100 times the

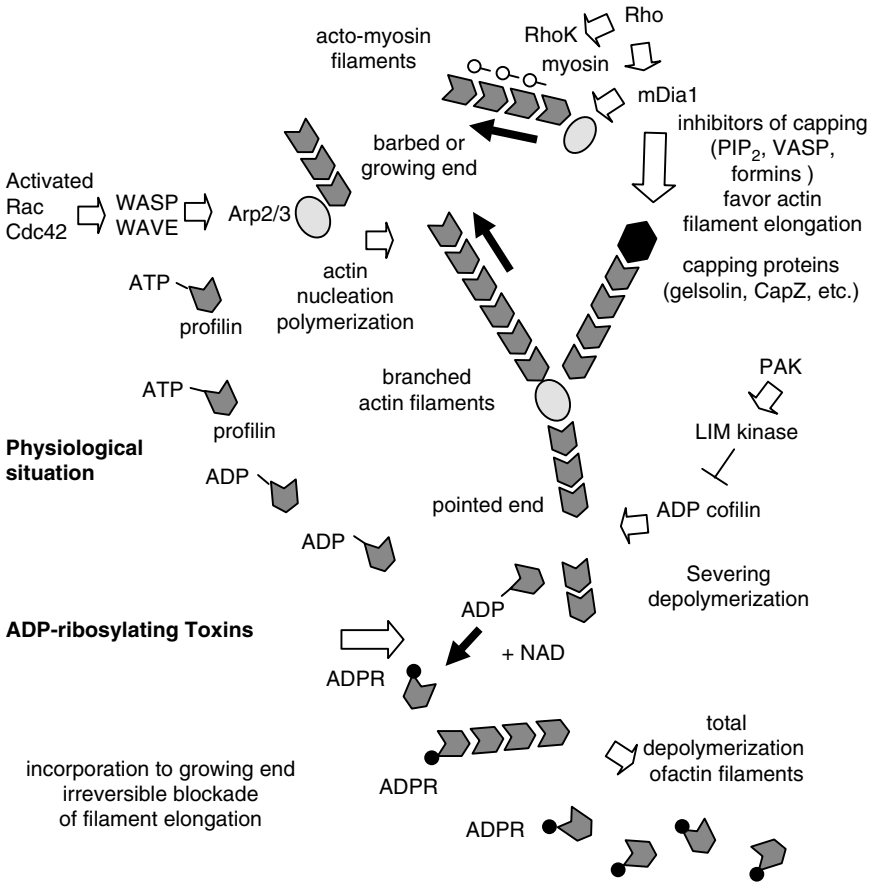



**FIGURE 9.2** Schematic representation of eucaryotic factors involved in actin nucleation and polymerization, as well as how ADP-ribosylating toxins inhibit actin polymerization by directly modifying actin monomers.

critical concentration necessary for polymerization) of polymerization-competent actin subunits. This results from several factors that include: (i) actin monomer-capping proteins, such as profilin, which recycle to ATP-actin and bind to barbed ends; (ii) barbed end capping proteins (i.e., gelsolin, CapZ), which quickly block polymerization of barbed ends and therefore concentrate available actin monomers to the few free barbed ends in response to a cellular or extracellular signal; and (iii) ADF/cofilin, which accelerate actin depolymerization, thus restoring the pool of ADP-actin monomers (Pollard and Borisy, 2003). Treadmilling is the continual process of adding and deleting actin monomers to the actin filament. The Arp2/3 complex also initiates actin polymerization from preexisting filaments leading to a branched actin network, since it remains attached to pointed ends and binds to the sides of actin filaments. Control of capping proteins at the barbed ends by inhibitory factors (i.e., $PIP_2$, VASP, formins) regulates actin filament polymerization. Another type of actin nucleator consists of the multidomain proteins called formins. These varying-sized molecules contain a highly conserved domain (FH2 or formin homology domain 2) that alone is sufficient to nucleate actin. In contrast to Arp2/3, formins generate straight filaments that bundle and associate with myosin to generate contractile structures such as stress fibers. Rho can activate the formin mDia1 and myosin through Rho-kinase (RhoK), thus leading to stress fiber formation (Zigmond, 2004).

Actin dynamics are regulated by numerous proteins in the cytosol that include: (i) actin monomer-binding proteins (i.e., thymosin β4, profilin); (ii) actin filament-binding proteins (i.e., gelsolin, CapG, CapZ ); (iii) actin filament-severing proteins; (iv) actin filament-bundling or cross-linking proteins (i.e., α-actinin, filamin, fimbrin, ABP120); and (v) actin depolymerizing proteins (i.e., ADF, cofilin) (Dos Remedios *et al*. 2002; Southwick and Purich, 2000). In addition, remodeling of the actin cytoskeleton in response to extracellular signals involves regulatory pathways encompassing small GTPases from the Rho family.

Rho proteins belong to the Ras superfamily, which are small (21–25 kDa) molecules that share structural homology and become activated only when bound to GTP. They are molecular switches that regulate various cellular processes including actin dynamics, endocytosis, gene transcription, cell-cycle progression, and differentiation. So far 20 members have been identified in the Rho-GTPase family (Burridge and Wennerberg, 2004), and the best-characterized molecules are Rho, Rac, and Cdc42, which respectively control the formation of stress fibers plus focal adhesions, membrane ruffling, and filopodia. Cycling between active and inactive forms of Rho-GTPases is regulated by at least three protein classes: (i) the guanine-nucleotide-exchange factors (GEFs), which promote exchange of bound GDP for GTP in response to an extracellular signal(s); (ii) the GTPase-activating proteins (GAPs), which enhance intrinsically low GTPase activity and thus inactivate Rho-GTPases; and (iii) the guanine-nucleotide-dissociation inhibitors (GDIs), which stabilize the GDP forms of Rho-GTPases in the cytosol (Van Aelst and Souza-Schorey, 1997; Bishop and Hall, 2000; Sah *et al*., 2000; Takai *et al*., 2001). Despite the evident diversity among GEF, GAP, and GDI molecules, each class shares a conserved mechanism of action (Vetter and Wittinghofer, 2001). Activated Rho-GTPases bind to membrane lipids via isoprenylated cysteines found in a Cys-Aliphatic residue-Aliphatic residue-X motif within the C-terminus, and then subsequently interact with their effectors. The conformational changes between GDP- and GTP-bound forms of Rho are localized within two surface loops, named *switch I* and *II*, which play an important role in GTP catalysis. Switch I of Rho-GTPases is the main region for interactions with effector molecules. Most Rac/Cdc42 effectors contain a conserved, GTPase-binding consensus site (CRIB), and many Rho effectors possess an N-terminal Rho effector homology domain (REM) composed of three leucine-zipper-like motifs. Effectors for Rho-GTPases often are Ser/Thr kinase, lipid kinase, lipase, or a scaffold protein (Bishop and Hall, 2000).

Bacteria have evolved various virulence factors, such as protein toxins and type-III secreted effectors, that can modulate the dynamic equilibrium of actin cytoskeletons via three routes: (i) modification of actin monomers leading to transient or permanent reorganization of the actin cytoskeleton that facilitates cell invasion, inhibition of phagocytosis, and/or alteration of cell barrier functions (Tables 9.1 and 9.2); (ii) regulation of Rho-GTPases by direct enzymatic modification or molecularly mimicking the eucaryotic regulatory proteins for Rho-GTPases (Tables 9.1, 9.2, and 9.3) and (iii) nucleation as well as formation of actin filaments promoting the intracellular mobility of bacteria and viruses (Table 9.3).

## BACTERIAL PROTEIN TOXINS TARGETING ACTIN

### *Bacillus* and *Clostridium* actin-ADP-ribosylating toxins

The actin-ADP-ribosylating toxins produced by *Bacillus* and *Clostridium* species share a common "A-B" structure consisting of two independent protein components not linked in solution by either covalent or non-covalent bonds (Table 9.1) (Barth *et al*., 2004). One protein represents a "B" component (~80–100 kDa) for cell-binding/membrane translocation, and the other is an enzymatic "A" component (~45 kDa). Three families can be distinguished among this binary toxin group. The Iota family includes *C. perfringens* iota toxin, *C. spiroforme* toxin, and a *C. difficile* toxin that differs from the large clostridal toxins designated as ToxA and ToxB. The C2 family corresponds to the C2 toxins produced by *C. botulinum* types C and D. These clostridial binary toxins are involved in necrotizing enteritis and diarrhea in animals, and occasionally are implicated in human diseases (Barth *et al*., 2004). The third family of bacterial ADP-ribosylating toxins that are likely specific for actin corresponds to the insecticidal binary toxins or vegetative insecticidal proteins (VIP) produced by *Bacillus cereus* and *Bacillus thuringiensis* (Han *et al*., 1999). Protein components within a family are highly related (80–85% identity among amino acid sequences), whereas the existing sequence identities between toxins from distinct families are noticeably less (29–40%). Protein components from the Iota family are immunologically cross-reactive and functionally complement each other to form chimeric toxins; however, this does not occur with either "A" or "B" component of C2 toxin (Popoff, 2000). Similar cross-reactivity and complementation experiments have not been reported for the VIP components with either the Iota or C2 family proteins.

The Iota and VIP family protein components are synthesized during exponential growth and contain a signal peptide, which facilitates secretion from the bacterium (Barth *et al*., 2004). In contrast, C2 toxin components lack a signal peptide, as they are produced during sporulation and subsequently released into the environment following sporangium lysis. All cell-binding components of these binary toxins are proteolytically activated via removal of a 20 kDa N-terminal peptide by different serine-type proteases (Barth *et al*., 2004). This in turn generates "activated" versions of the cell-binding component that form homoheptamers in

**TABLE 9.1** Secreted bacterial toxins that modulate the actin cytoskeleton

| Toxins | Pathogens | Cellular targets | Enzymatic activity | Effects | Ref |
|---|---|---|---|---|---|
| **Binary toxins** | | | | | |
| Iota | *Clostridium perfringens* E | cellular and muscular G-actin | ADP-ribosylation | depolymerization of actin filaments, alteration of cell barriers | (Barth *et al*., 2004) |
| CST | *Clostridium spiroforme* | | | | |
| CDT | *Clostridium difficile* | | | | |
| C2 toxin | *Clostridium botulinum* C, D | cellular G-actin | | | |
| VIP | *Bacillus thuringiensis* | | | | |
| | *Bacillus cereus* | | | | |
| **C3 and C3-like enzymes** | | | | | |
| C3 | *Clostridium botulinum* C, D | RhoA, B, C | ADP-ribosylation | depolymerization of actin filaments | (Barbieri *et al*., 2002) |
| C3lim | *Clostridium limosum* | | | | |
| C3cer | *Bacillus cereus* | | | | |
| EDIN | *Staphylococcus aureus* | RhoA, B, C, E | | | |
| **Large clostridial toxins** | | | | | |
| ToxA | *Clostridium difficile* | RhoA, Rac, Cdc42 | UDP-glucosylation | depolymerization of actin filaments, alteration of cell barriers | (Just *et al*., 2000) |
| ToxB | *Clostridium difficile* | RhoA, Rac, Cdc42 | UDP-N-acetyl-glucosylation | | |
| α–novyi | *Clostridium novyi* | RhoA, Rac, Cdc42 | | | |
| LT | *Clostridium sordellii* | Rac, Ras, Rap, Ral | UDP-glucosylation | | |
| HT | *Clostridium sordellii* | RhoA, Rac, Cdc42 | | | |
| **Dermonecrotizing toxins** | | | | | |
| CNF1, CNF2 | *Escherichia coli* | Rho, Rac, Cdc42 | Deamidation | alteration of cell barrier, stimulation of phagocytosis | (Boquet, 2001) |
| DNT | *Bordetella pertussis* | Rho, Rac (Cdc42) | Deamidation | | |
| | *Bordetella parapertussis* | | Polyamination | | |
| | *Bordetella bronchiseptica* | | | | |
| **Enterotoxins** | | | | | |
| CPE | *Clostridium perfringens* | claudin, occludin | pore formation | | (McClane, 2001) |
| Fragilysin/BFT | *Bacteroides fragilis* | E-cadherin | protease | disorganization of actin filaments and alteration of cell barrier | (Sears, 2001) |
| ZOT | *Vibrio cholerae* | PKCα | actin polymerization TJ opening | | (Wang *et al*., 2000) |
| | | PLC activation | | | (Fullner and Mekalanos, 2000) |
| RTX | *Vibrio cholerae* | G-actin | actin monomer cross-linking | | |
| HA/P | *Vibrio cholerae* | occludin | proteolysis | | (Wu *et al*., 2000) |

solution and/or on cell membranes. The enzymatic component (Ia) of iota toxin also requires the removal of 9 to 11 N-terminal residues for activity (Gibert *et al*., 2000), a unique process not yet elucidated for the other bacterial ADP-ribosyltransferases that target actin. To date, for the Iota, C2, and VIP family of toxins, it is unknown how many molecules of "A" bind to a heptameric form of "B." However, studies with closely related binary toxins produced by *Bacillus anthracis* (i.e., lethal and edema toxins) suggest a 3:7 mole ratio of A:B (Mogridge *et al*., 2002b).

The binding components of actin-ADP-ribosylating toxins share an overall sequence identity of 31–43% with the "B" component (protective antigen or PA) of *Bacillus anthracis* edema and lethal toxins. The PA crystal structure has been resolved at 2.1 angstroms and the molecule clearly possesses four distinct domains (Petosa *et al*., 1997). Following proteolysis and homoheptamer formation, the N-terminal domain (domain 1'; residues 1–249) of PA contains an exposed docking site for an enzymatic component (edema factor or lethal factor) (Cunningham *et al*., 2002). The edema and lethal factors share a common Val-Tyr-Tyr-Glu-Ile-Gly-Lys motif that is necessary for docking to PA. The C-terminal domain of PA (domain 4; residues 597–735) is involved in recognizing cell-surface protein receptors, recently

identified as tumor endothelium marker 8 and capillary morphogenesis gene 2 (Petosa *et al*., 1997; Bradley *et al*., 2001). Domain 3 (residues 488–596) is responsible for homoheptamer formation, which is subsequently important for effective docking of the edema and lethal factors to PA on the cell surface. Domain 2 (residues 250–487) contains long β-strands that form a classic "Greek key" motif that plays a central role in membrane insertion and channel formation. A flexible, amphipathic loop (amino acids 302–325) in PA forms a β-hairpin structure that within the PA heptamer collectively generates a β-barrel, which inserts into the membrane and facilitates translocation of partially unfolded enzymatic component(s) into the cytosol (Cunningham *et al*., 2002; Mogridge *et al*., 2002a; Mogridge *et al*., 2002b; Abrami *et al*., 2003).

The overall PA structure and domain organization are likely conserved among binding components of the actin-ADP-ribosylating toxins from other *Clostridium* and *Bacillus species*. Results generated by various techniques suggest that the C-terminus of each binding component from iota (Ib) and C2 (C2-II) toxins, corresponding to domain 4 of PA, is responsible for cell-surface recognition (Blöcker *et al*., 2000; Marvaud *et al*., 2001). Lower levels of a conserved amino acid sequence (less than 10% homology) between domains 4 of Ib and C2-II suggest that each toxin recognizes distinct receptors, which include a membrane protein for iota toxin (Stiles *et al*., 2000), and an Asn-linked glycan containing the α-D-mannoside-β1, 2-*N*-acetyglucosamine motif for C2 toxin (Eckhardt *et al*., 2000). The N-terminal region of either mature Ib or C2-II is required for Ia and C2-I docking, respectively (Barth *et al*., 1998; Marvaud *et al*., 2001). Similar work has not been done with the *C. difficile* and *C. spiroforme* binary toxins, or *Bacillus* VIP.

Crystal structures of the VIP (VIPII) and iota toxin (Ia) enzymatic components reveal two distinct, but related, structural domains that were probably derived by duplication of an ancestral gene (Figure 9.3). Each domain consists of a perpendicular packing of five mixed, β-sheet strands against a three stranded, antiparallel β-sheet flanked by four consecutive helices. The C-terminal domain, which shares homology with *C. botulinum* C3 ADP-ribosyltransferase specific for Rho-GTPases, encompasses the catalytic site; whereas the N-terminus interacts with heptameric forms of the binding component that subsequently facilitate enzyme entry into the cytosol (Han *et al*., 1999; Tsuge *et al*., 2003). The Ia segment encompassing amino acids 129–257 corresponds to a compact core within the N-domain and mediates docking with heptameric Ib (Marvaud *et al*., 2002); whereas the C2-I domain that binds to heptameric C2-II has been mapped to residues 1–87, which corresponds to the four α-helices located upstream from the N-domain core of VIPII (Barth *et al*., 2002).

## Mode of action

The proteolytically-activated binding components of *Bacillus* and *Clostridium* binary toxins are each ~80 kDa, or ~60 kDa for C2II as well as PA, and trigger internalization of enzymatic component(s) into cells by receptor mediated endocytosis (Barth *et al*., 2004). Mature binding components first recognize specific, cell-surface receptors, then subsequently form homoheptameric "B" structures and small ion permeable

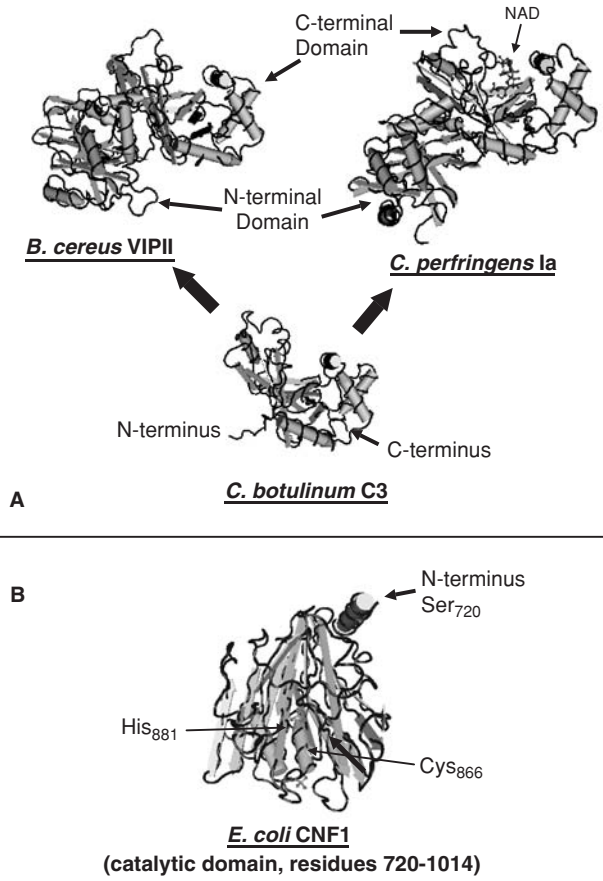



**FIGURE 9.3** Structures of the enzymatic components (VIPII and Ia) from actin ADP-ribosylating toxins, the Rho ADP-ribosylating enzyme C3 (A), and enzymatic domain of the Rho-deamidating toxin CNF (B). Enzymatic components of VIP and iota toxins each contain two similar domains that probably evolved from gene duplication. One domain (C-terminal) retains the enzymatic site that is highly related to the entire C3 molecule, which itself contains only an enzymatic domain devoid of any recognized cell-binding domain. In contrast, the deamidating domain of CNF is structurally unrelated to those of actin or Rho ADP-ribosylating toxins.

channels that facilitate enzyme transport into the cytosol via acidic endocytic vesicles (Schmid *et al*., 1994; Barth *et al*., 2000; Bachmeyer *et al*., 2001; Knapp *et al*., 2002). In contrast to PA, which is proteolytically activated either in solution by serine-type proteases or on the cell surface by furin or furin-like proteases via a unique Arg-Lys-Lys-Arg cleavage site, Ib and C2-II are exclusively processed in solution by serine-type proteases such as trypsin or alpha-chymotrypsin (Barth *et al*., 2004). After activation in solution, the PA, Ib, and C2-II molecules can also form homoheptamers that subsequently bind to cells. Proteolytically unprocessed Ib and C2-II also bind to cell-surface receptors, but they neither oligomerize nor mediate docking/entry of the enzymatic components into cells (Gibert *et al*., 2000; Stiles *et al*., 2000; Stiles *et al*., 2002).

Enzymatic components from the Iota toxin family can ADP-ribosylate all cellular and muscular isoforms of actin, whereas C2 toxin is more discriminating and only interacts with cytoplasmic and smooth muscle γ-actin. The ubiquitous nicotinamide adenine dinucleotide (NAD) molecule, commonly used by cells for normal reduction/oxidation reactions necessary for homeostasis, represents the source of ADP-ribose for specifically modifying actin monomers (G-actin), but not polymerized F-actin, at Arg177 (Vandekerckhove *et al*., 1987). Actin monomers complexed to gelsolin also represent an appropriate substrate for ADP-ribosylation by these bacterial enzymes. The targeted Arg177 residue is located at the actin-actin binding site, which is buried within an actin filament, and ADP-ribosylation on this part of G-actin prevents nucleation and subsequent polymerization. Moreover, ADP-ribosylated actin acts as a capping protein that binds to the barbed end of an actin filament, thus inhibiting further addition of unmodified actin monomers. Actin filaments depolymerize at the pointed end and the released actin monomers are immediately modified with ADP-ribose (Figure 9.2). In addition, ADP-ribosylation inhibits the intrinsic ATPase activity of actin. Microinjection of ADP-ribosylated actin monomers into cells induces the same effects (i.e., disassembly of actin filaments) as that seen after C2 or iota intoxication (Kiefer *et al*., 1996; Reuner *et al*., 1996; reviewed in Aktories, 2000). To date, the specific substrate(s) of VIPII within insect cells has not been determined experimentally, but the sequence and conformation are highly suggestive of an actin-modifying protein.

### Effects on cell barriers

The most prominent effects of clostridial ADP-ribosylating toxins include depolymerization of actin filaments and a subsequent increase in the G-actin pool. Intermediate filaments are also disorganized, unlike the microtubules, and the overall effects upon cells involve overt rounding and death (Aktories, 2000; Ohishi, 2000). For polarized monolayers of CaCo-2 cells, iota toxin can bind and then induce depolymerization of the actin cytoskeleton from either the apical or basolateral surfaces (Richard *et al*., 2002). The TJs and AJs are subsequently disorganized, resulting in decreased transepithelial resistance and increased paracellular permeability of small, as well as large, molecules that include $^{14}$C-mannitol and FITC dextrans ranging from 4.4 to 35 kDa (Figure 9.4) (Petit *et al*., 2001). Experimentally, C2 toxin is dermonecrotic like iota toxin and induces hemorrhagic enteritis in mice (Ohishi and Odagiri, 1984). In rabbit lungs, the C2 toxin also causes edema and alteration of endothelial cell membranes (Ermert *et al*., 1997). The massive edema and hypotonic shock observed in animals injected with C2 toxin probably results from deterioration of the vascular endothelium barrier (Aktories and Koch, 1995).

## TYPE-III SECRETION OF ACTIN ADP-RIBOSYLATING FACTORS

Non-typhoid strains of *Salmonella*, commonly associated with a major form of food poisoning that may include severe systemic infections, contain a 90 kb plasmid required for growth in macrophages and monocytes that harbors four *spv* (*Salmonella* plasmid virulence) genes (A, B, C, D) in an operon regulated by SpvR. Among these genes, *spvB* encodes a 65 kDa protein (SpvB) essential for virulence. Recombinant SpvB specifically ADP-ribosylates non-muscle actin (Table 9.2 and Figure 9.2), and is the first ADP-ribosyltransferase described as a virulence factor for any intracellular pathogen (Lesnick *et al*., 2001). The C-terminal domain of SpvB shares conserved residues and overall sequence homology with the ADP-ribosylating site of binary toxins produced by *Clostridium* (Iota and C2) or *Bacillus* (VIP, 25% with *B. cereus* VIPII), which specifically modify actin monomers. The Ser-Thr-Ser motif, flanked by a downstream Glu-X-Glu and upstream Arg, represents some of the common residues evident in ADP-ribosyltransferases from both Gram-negative and Gram-positive bacteria. CHO cells transfected with the *spvB* gene undergo actin filament destruction, and SpvB is crucial for virulence in a subcutaneous mouse model, as evidenced by mutant strains of *Salmonella typhimurium* lacking a functional *spvB* gene (Matsui *et al*., 2001). The N-terminal domain of SpvB is involved in secretion and shares 47% sequence homol-

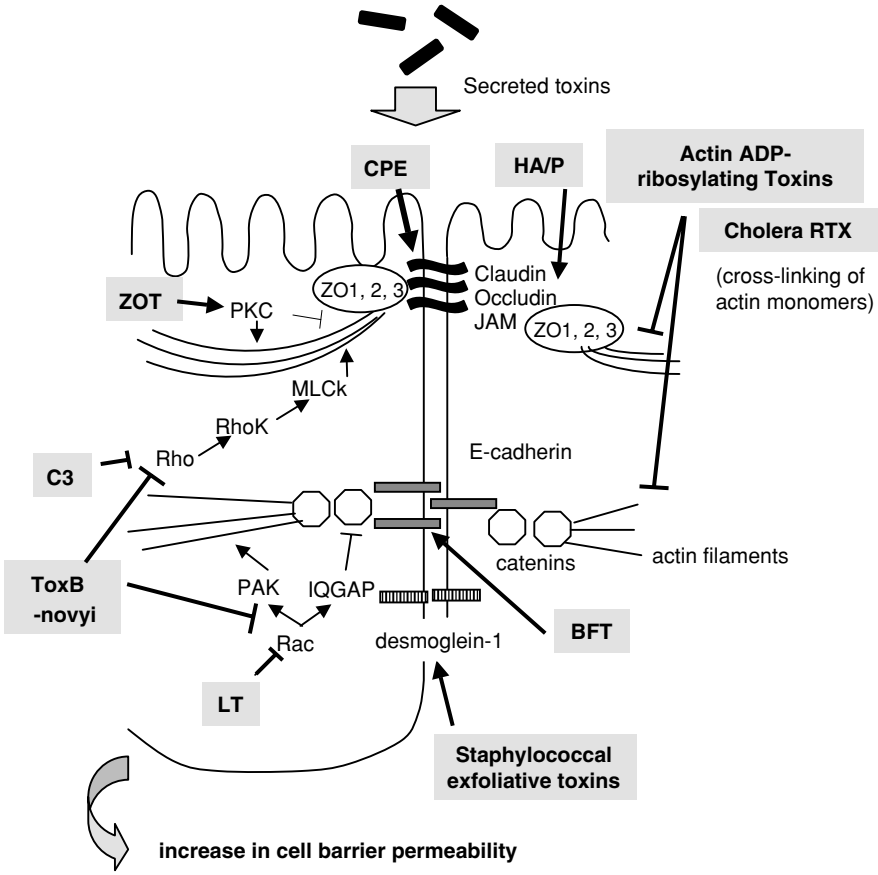



**FIGURE 9.4** Organization of the actin cytoskeleton and intercellular junctions, as well as toxins which modify the actin cytoskeleton and/or intercellular junction components. CPE, *C. perfringens* enterotoxin which binds to claudin(s) and incorporates occludin into a large complex (McClane, 2001); HA/P, haemagglutinin/protease from *V. cholerae* which degrades occludin and disturbs ZO-1 as well as actin filament organization (Wu *et al.*, 2000); ZOT, *V. cholerae* zonula occludin toxin which induces actin polymerization and ZO-1 disengagement from occludin as well as claudin (Wang *et al.*, 2000); RTX, *V. cholerae* repeats-in-toxin which cross-links actin monomers and causes depolymerization of actin filaments (Fullner and Mekalanos, 2000); BFT, *B. fragilis* enterotoxin which cleaves cadherin (Sears, 2001); staphylococcal exfoliative toxins which proteolytically cleaves desmoglein-1 (see chapter 56); LT, *C. sordellii* lethal toxin which glucosylates Rac leading to inactivation; ToxB, *C. difficile* toxin B which glucosylates Rho leading to inactivation; α-novyi, *C. novyi* toxin which transfers N-acetylglucosamine to Rac leading to inactivation (Schirmer and Aktories, 2004).

ogy with the insecticidal toxin TcaC produced by *Photorhabdus luminescens*, a motile Gram-negative bacillus of the Enterobacteriaceae family that also has a type-III secretion system and inhabits the gut of nematodes, which are themselves subsequently consumed by insects that become intoxicated by TcaC (Chattopadhyay *et al.*, 2004). To date, the exact mode of action for TcaC is unknown, but it may suppress phagocytosis by hemocytes via the actin cytoskeleton.

Like *Salmonella* SptP (described below), SpvB also reverses the effects of virulence factors that induce membrane ruffling and reorganization of the actin cytoskeleton for bacterial entry, thus permitting infected cells to regain their normal architecture after invasion. Although SpvB may be injected into host cells like SptP via a type-III secretion system, it has been shown that SpvB is secreted into growth medium by *Salmonella* mutants defective in type-III secretion (Gotoh *et al.*, 2003). The *spvB* gene is expressed 6 h after bacterial entry into epithelial cells or macrophages, and SpvB-dependent cytotoxicity/apoptosis is evident 10–12 h post infection (Lesnick *et al.*, 2001; Kurita *et al.*, 2003). In similar fashion, the fish pathogen *Aeromonas salmonicida* represents another member of the Enterobacteriaceae that produces an essential, type-III secreted virulence factor recently identified as a 50 kDa, ADP-ribosyltransferase designated as AexT (Burr *et al.*, 2003). Although the target substrate has not been clearly defined to date, AexT shares high sequence identity (60%) and epitopes with GAP ADP-ribosyltransferases, ExoS and ExoT, produced by *Pseudomonas aeruginosa*.

Finally, similar ADP-ribosyltransferase genes have recently been identified in the genome of *Menigococcus*

(NarE) and *Listeria monocytogenes* by a novel, computer-based approach based upon pattern recognition and predicted secondary structure (Masignani *et al*., 2004).

## TOXINS INACTIVATING RHO-GTPASES

### C3 and C3-like enzymes

Approximately 20 years ago, exoenzyme C3 was first identified from *Clostridium botulinum* types C and D as an ADP-ribosyltransferase specific for Rho protein (Aktories *et al*., 1987; Rubin *et al*., 1988). Two isoforms, C3bot1 and C3bot2, have subsequently been characterized and share 60% sequence identity. C3 and related C3-like molecules are ~25 kDa, basic (pI >9) proteins that are also produced by other Gram-positive bacteria, such as *Clostridium limosum* (C3lim), *B. cereus* (C3cer), and *Staphylococcus aureus* (Wilde *et al*., 2001). The C3 related enzyme produced by *S. aureus* is called *epithelial differentiation inhibitor* (EDIN), or C3stau, and consists of three isoforms (A, B, C) sharing ~35% sequence identity with the *C. botulinum* C3 molecule (Table 9.1). The EDIN-producing strains of *S. aureus* (~7.8% of all clinical isolates) are preferentially obtained from skin suppurations as well as other infection sites, versus only 3.7% of *S. aureus* isolated from healthy nasal carriers, thus suggesting that EDIN may be a virulence factor (Czech *et al*., 2001).

### Structure and enzymatic activity

C3 enzymes are secreted from bacteria into the extracellular milieu and possess a catalytic domain, but curiously lack binding and translocation domains that would permit targeted entry into cells. Crystallography data of C3 molecules from *S. aureus* and *C. botulinum* reveal a core structure containing five antiparallel β-strands packed against three-stranded antiparallel β-sheets that are flanked by four consecutive α-helices (Han *et al*., 2001; Evans *et al*., 2003). Interestingly, the C3 conformation is strikingly similar to the actin-specific, catalytic domains found in *B. cereus* VIPII and *C. perfringens* Ia (Figure 9.3) (Han *et al*., 1999; Tsuge *et al*., 2003).

Although there is no significant overall sequence homology with other ADP-ribosylating toxins, C3 retains the conserved NAD binding site and catalytic pocket consisting of an α-helix (α3; residues 83–93) bent over two antiparallel β-sheets that form a central cleft. The amino acids that have an essential role in ADP-ribosylation are conserved and consist of a hydrophobic segment containing the $_{174}$Ser-Thr-Ser$_{176}$ motif, flanked by Arg128 and Glu214 (Han *et al*., 2001). Additionally, Glu214 plays a pivotal role in catalysis of NAD and transfer of the ADP-ribose group onto Asn41 of Rho. C3 and the clostridial binary toxins differ from other ADP-ribosylating toxins by containing two adjacent protruding turns, designated as an ADP-Ribosylating toxin Turn-Turn (ARTT) motif consisting of Phe/Tyr-X-X-Gln/Glu-X-Glu involved in specific substrate recognition. However, the C3 and EDIN molecules that specifically modify Rho-GTPases contain a conserved Gln212 in turn 2, whereas the actin-ADP-ribosylating toxins have a Glu at this position (Han *et al*., 1999; Han *et al*., 2001). The ARTT-containing loop adopts a conformation upon NAD binding that permits transition of Gln212 from a buried, to a solvent-exposed, position. This indicates that C3 acts in three distinct steps that include NAD binding, Rho binding, and finally Rho ADP-ribosylation (Ménétrey *et al*., 2002). All C3 exoenzymes recognize RhoA, B, or C, and *S. aureus* EDIN also modifies RhoE (Wilde *et al*., 2001).

Rho-GDP is a preferred substrate for C3 (Habermann *et al*., 1991), as Rho-Asn41 is solvent accessible in the GDP structure (Wei *et al*., 1997). In contrast, the Asn41 residue of Rho found in a Rho-GDI complex is hidden and thus resistant to C3-mediated ADP-ribosylation (Bourmeyster *et al*., 1992). ADP-ribosylation of Rho-Asn41 by C3 does not impair GDP/GTP exchange, does not affect intrinsic GAP-stimulated GTPase activity, and does not impinge upon Rho interaction with its effectors (Ren *et al*., 1996; Sehr *et al*., 1998). However, ADP-ribosylation of Rho does block translocation to the membrane where it is required for activation and interactions with its effectors. Finally, ADP-ribosylated Rho can reassociate more efficiently with GDI than unmodified Rho, thus causing an accumulation of inactive Rho in the cytosol (Figure 9.5) (Fujihara *et al*., 1997; Ohishi, 2000).

### Effects on cell barriers

The enzymatic effects of C3 have been widely used to study the function of Rho, and the results from various groups implicate this GTPase in establishing, as well as maintaining, adhesive structures whose regulation may vary between cell types. For example, C3 induces cell rounding and destruction of actin filaments in epithelial and fibroblast cells, but there is little disorganization of cortical actin (Chardin *et al*., 1989). In polarized T84 cells, C3 profoundly alters the apical actin cytoskeleton, distribution of occludin, ZO-1 and claudin-1 found in TJs, as well as increases cell barrier permeability; however, there is no perturbation of the AJs (Nusrat *et al*., 1995; Walsh *et al*., 2001). C3 microinjected into MDCK cells causes the disappearance of

**TABLE 9.2** Type -III and type-IV secretion factors that modulate the actin cytoskeleton for cell invasion or escape from phagocytosis

| Type-III virulence factors | Pathogens | Cellular targets | Biochemical activity | Effects | Ref |
|---|---|---|---|---|---|
| SopE1/SopE2 | *Salmonella typhimurium* | Cdc42, Rac | GDP-GTP exchange factor | actin polymerization cell invasion | (Friebel *et al*., 2001) |
| SopB/SigD | *Salmonella typhimurium* | Cdc42 | inositol phosphatase | cell invasion | (Zhou *et al*., 2001) |
| SptP | *Salmonella typhimurium* | Cdc42, Rac | GTPase activating protein | actin filament dissociation rearrangement of actin cytoskeleton | (Stebbins and Galan, 2000) |
| SipA | *Salmonella typhimurium* | F-actin | unknown actin filament cross-linking | actin polymerization cell invasion | (Lilic *et al*., 2003) |
| SipC | *Salmonella typhimurium* | actin | unknown | actin nucleation cell invasion | (Hayward and Koronakis, 1999) |
| SpvB | *Salmonella typhimurium* | G-actin | ADP-ribosylation | actin filament dissociation rearrangement of actin cytoskeleton | (Lesnick *et al*., 2001) |
| ExoS | *Pseudomonas aeruginosa* | Rho, Rac, Cdc42 Rho, Rac, Cdc42, Ras, Rap | GTPase activating protein ADP-ribosylation | actin filament dissociation antiphagocytosis | (Barbieri *et al*., 2002) |
| ExoT | *Pseudomonas aeruginosa* | Rho, Rac, Cdc42 | GTPase activating protein ADP-ribosylation | actin filament dissociation antiphagocytosis | (Barbieri *et al*., 2002) |
| IpaA | *Shigella flexneri* | vinculin | unknown | actin filament dissociation rearrangement of actin cytoskeleton | (Tran Van Nhieu *et al*., 2000) |
| IpaC | *Shigella flexneri* | actin | unknown | actin nucleation and polymerization cell invasion | (Tran Van Nhieu *et al*., 2000) |
| YopE | *Yersinia enterocolitica* | Rho, Rac, Cdc42 | GTPase activating protein | actin filament dissociation antiphagocytosis | (Black and Bliska, 2000) |
| YopT | *Yersinia enterocolitica* | Rho, Rac, Cdc42 | cysteine protease | actin depolymerization antiphagocytosis | (Shao *et al*., 2002) |
| YopH | *Yersinia enterocolitica* *Yersinia pseudotuberculosis* | p125FAK, p130CAS | tyrosine phosphorylation | focal adhesion disruption antiphagocytosis | (Cornelis, 2002) |
| YopO/YpkA | *Yersinia enterocolitica* | Rho, Cdc42 | Ser/Thr kinase | actin depolymerization antiphagocytosis | (Cornelis, 2002) |
| Tir | Enteropathogenic *Escherichia coli, Citrobacter rodentium* | Nck | tyrosine kinase | actin polymerization actin pedestal formation | (Campellone and Leong, 2003) |
| IpgD | *Shigella flexneri* | phosphatidyl inositol 4, 5-biphosphate | inositol 4-phosphatase | actin rearrangement cell invasion | (Niebuhr *et al*., 2002) |
| $EspF_u$ | *Enterohemorrhagic E. coli* | N-WASP | unknown | actin polymerization actin pedestal formation | (Campellone *et al*., 2004) |
| CT456 | *Chlamydia trachomatis* | unknown | unknown | actin pedestal formation | (Clifton *et al*., 2004) |
| CagA (type IV secretion) | *Helicobacter pylori* | Ezrin SHP2 activation oncoprotein | induces phosphatase activity | | (Selbach *et al*., 2004) |
| type IV factor | *Helicobacter pylori* | Rac, Cdc42 activation | unknown | actin pedestal formation | (Naumann and Crabtree, 2004) |

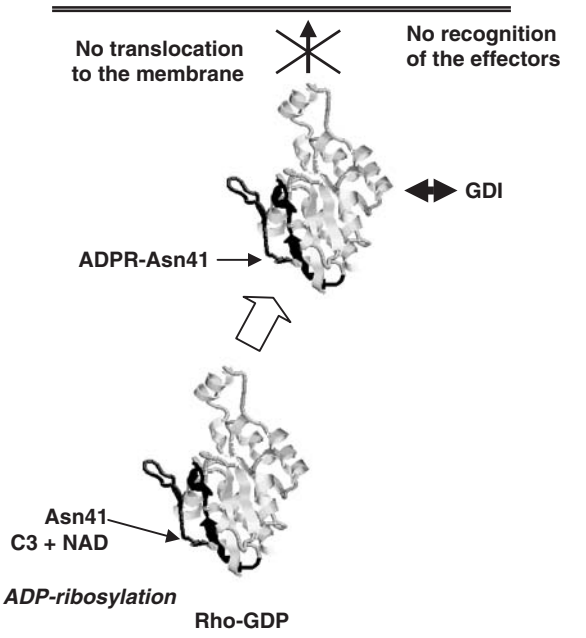



**FIGURE 9.5** Schematic mode of action for the Rho-inhibiting C3 enzyme via ADP-ribosylation.

actin filaments at cell-cell adhesion sites, as well as alters formation of TJs and AJs (Takaishi *et al*., 1997). In addition, C3 inhibits association of the ERM (ezrin, radixin, and moesin) family of proteins and vinculin with the plasma membrane, which is necessary for developing the basal edge and focal adhesions of cells (Kotani *et al*., 1997). Overall, this information indicates that Rho plays a central role in forming and maintaining TJs. Consistently, RhoK is a major downstream effector that controls TJ formation through monophosphorylation of the myosin light chain and contraction of the perijunctional actomyosin ring (Turner *et al*., 1997). However, as opposed to Rho inactivation by C3, inhibition of RhoK with Y27632 induces a redistribution of TJ molecules without completely abolishing the monolayer integrity (Walsh *et al*., 2001). Another mechanism independent of RhoK and the cytoskeleton could also be involved in TJ formation and phosphorylation of occludin, as measured by transepithelial electrical resistance (TER) (Hirase *et al*., 2001; Schneeberger and Lynch, 2004).

Effects of Rho on AJs, the latter structure being found underneath TJs and involved in cell-cell contact, gene expression, and cell differentiation via numerous (n >30) cadherin family proteins (Perrez-Moreno *et al*., 2003), depend upon the cell type and junctional maturity. For example, C3 disrupts E-cadherin staining of keratinocytes and MDCK cells, but not that in T84 or human umbilical endothelial cells (Nusrat *et al*., 1995; Braga *et al*., 1997; Takaishi *et al*., 1997; Braga *et al*., 1999). Integrin localization at cell-cell contact sites is not modified in keratinocytes treated with C3 (Braga *et al*., 1999). Other experiments show that C3 does not alter AJs in MDCK cells transfected with a dominant positive Rac, thus suggesting that AJs are mainly regulated by Rac (Takaishi *et al*., 1997). The Rho molecule probably controls E-cadherin intercellular junctions indirectly through rearranging the actin cytoskeleton (Fukata and Kaibuchi, 2001).

In contrast to epithelial cells, C3 disassembles actomyosin filaments and focal adhesions formed by endothelial cells, but it does not alter the intercellular junctions or endothelial permeability. In fact, C3 prevents the endothelial permeability induced by vasoactive agents like thrombin and histamine, thus indicating Rho involvement in this process. C3 also blocks the increased actomyosin contractility leading to cell retraction, intercellular gap formation, and the loss of TJs and AJs caused by thrombin as well as histamine (Figure 9.4) (Vouret-Craviari *et al*., 1998; Carbajal and Schaeffer, 1999; Wojciak-Stothard *et al*., 2001).

## CLOSTRIDIAL GLUCOSYLATING TOXINS

The glucosylating toxins produced by various clostridial species, also known as the large clostridial toxins, are 250–300 kDa single-chain proteins that include *C. difficile* toxins A (ToxA) and B (ToxB), *C. sordellii* lethal toxin (LT), and hemorrhagic toxin (HT), as well as *C. novyi* α toxin (α-novyi) (Table 9.1) (Schirmer and Aktories, 2004). Glucosylating toxins represent the main virulence factors of these *Clostridium* species, commonly involved in intestinal diseases and/or myonecrosis. *C. difficile* can cause pseudomembranous colitis and enteritis that often follow antibiotic treatment, which represents the most prevalent nosocomial form of diarrheal disease in hospitals (see chapter 22). *C. sordellii* and *C. novyi* both appear as common causative agents of gas gangrene in humans and animals. Additionally, *C. sordellii* is involved in hemorrhagic enteritis of cattle as well as enterotoxemia in sheep, and *C. novyi* can induce bovine hepatotoxemia (Songer, 1996).

The glucosylating toxins of *Clostridium* contain three functional domains. The C-terminus displays multiple repeated sequences that are homologous with peptide segments from streptococcal glucosyltransferases and recognize cell surface receptor(s). For example, the corresponding domain in ToxA recognizes a trisaccharide (Gal-α1-3Gal-β1-4GlcNac) motif, and a cell surface receptor found in rabbit ileal brush borders was subsequently

identified as the glycoprotein sucrase-isomaltase; however, this protein is not expressed in the human colon. Clearly, further work needs to be done to identify the receptor for ToxA that is pertinent in the human intestinal tract (Just *et al*., 2000). The central part of these toxins contains a hydrophobic segment that probably mediates translocation across a cell membrane (Pfeifer *et al*., 2003). An enzymatic site characterized by a Asp-X-Asp motif surrounded by hydrophobic regions, and the substrate recognition site, are both localized within the first 546 N-terminal residues of ToxB and 659 N-terminal residues of ToxA. ToxB and LT are highly related (76% amino acid sequence identity), but there is less identity (48–60%) evident with ToxA and α-novyi (Aepfelbacher *et al*., 2000).

## Mode of action

Large clostridial toxins enter cells by receptor-mediated endocytosis. The cytotoxic effects are blocked by inhibitors (i.e., monensin, bafilomycin A1, ammonium chloride) of endosomal and lysosomal acidification, a process that can be circumvented by an extracellular acidic pulse that facilitates direct toxin entry into the cytosol from the outer surface of the cell membrane (Qa'dan *et al*., 2000; Barth *et al*., 2001; Qa'dan *et al*., 2001). This indicates that the large clostridial toxins translocate from early endosomes following an acidification step. At low pH, ToxB and LT induce channel formation in cell membranes and artificial lipid bilayers, which correlates with a concomitant increase in hydrophobicity as determined by fluorescence methods (Qa'dan *et al*., 2000, 2001). This probably involves a conformational change and subsequent insertion of toxin into the membrane, possibly mediated by a hydrophobic peptide segment(s) located within the central domain. Although not clearly delineated, it is likely that a proteolytically derived N-terminal fragment containing the active site is translocated into the cytosol (Pfeifer *et al*., 2003).

Large clostridial toxins catalyze the glucosylation of 21 kDa G-proteins using UDP-glucose, with an exception being α–novyi, which utilizes UDP-*N*-acetylglucosamine as co-substrate. These enzymes transfer one molecule of glucose or *N*-acetylglucosamine to the hydroxyl group of an acceptor amino acid, such as Thr37 of Rho or Thr35 of Rac, Cdc42, and Ras proteins (Just *et al*., 1995; Böhmer *et al*., 1996). Rho complexed to GDI is not a substrate for glucosylation, and modified Rho does not bind to GDI (Genth *et al*., 1999). ToxA and ToxB glucosylate Rho, Rac, and Cdc42, whereas LT modifies Rac, Cdc42, and several Ras proteins (i.e., Ras, Rap, Ral). Some *C. difficile* and *C. sordellii* strains respectively produce variant ToxB or LT molecules that differ in activity towards specific substrates.

The conserved, glucosylated Thr37/35 residues are located on a loop within switch I of Rho-/Ras-GTPases and exposed only on the surface of GDP-bound GTPase (Figure 9.6). Therefore, GDP forms of GTPases represent a preferred substrate for glucosylating

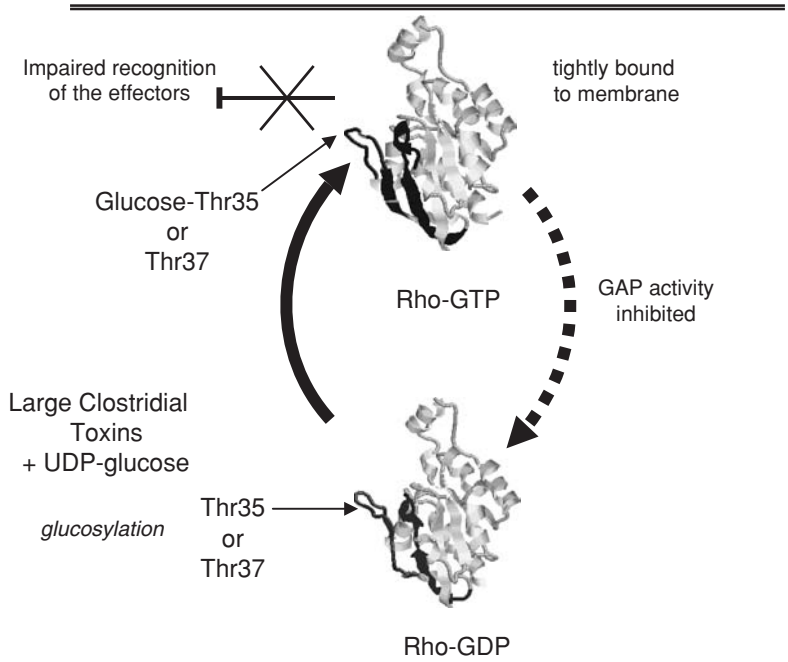



**FIGURE 9.6** Schematic mode of action for the large clostridial toxins that inhibit Rho-GTPases via glucosylation.

toxins. The Thr37/35 residues are involved in coordinating $Mg^{++}$ and subsequent binding of the β- and γ-phosphates of GTP. Nucleotide binding of glucosylated Ras by LT is not grossly altered, but the GEF activation of GDP forms is decreased (Essler *et al*., 1998). Glucosylation of Thr35 prevents recognition of the downstream effector, blocking the G-protein in an inactive form (Essler *et al*., 1998). Analysis of the crystal structure of Ras, modified by LT, shows that glucosylation prevents switching towards a GTP-bound conformation that exposes the effector loop necessary for interaction with an effector molecule like Raf (Vetter *et al*., 2000). Similar results were found with RhoA following glucosylation by ToxB (Sehr *et al*., 1998). In addition, glucosylation of the GTPase slightly reduces intrinsic GTPase activity and completely inhibits GAP-stimulated hydrolysis of GTP (Essler *et al*., 1998), leading to an accumulation of the GTP-bound form of Rho near the membrane where it binds to an unknown protein of ~70 kDa in a saturable, competitively inhibited manner. Glucosylated Rho does not cycle between a GDP- and GTP-bound form or the cytosol and membrane (Genth *et al*., 1999), and the crucial inactivation step of Ras-/Rho-GTPases by glucosylation consists of GTPase uncoupling from effector molecules.

## Effects on cell barriers

ToxB induces loss of actin stress fibers and reorganization of focal adhesions accompanied by cytoplasmic retraction and cell rounding, whereas long protrusions radiating around the cell yield a particular "actinomorphic" morphology (Ottlinger and Lin, 1988). ToxA and α-novyi cause similar cell alterations, but LT produces cell rounding without branched, membrane protrusions. In contrast to C3, large clostridial toxins modify more than one GTPase that renders more difficult the interpretation of cellular effects. Such effects could be linked to the simultaneous inhibition of several GTPase pathways and activities other than GTPase glucosylation attributed to the large clostridial toxins.

Alteration of the actin cytoskeleton is dependent upon intracellular UDP-glucosylation by *C. difficile* and *C. sordellii* toxins, since a cell line deficient in UDP-glucose is resistant to these toxins (Chaves-Olarte *et al*., 1996). Disruption of actin filaments by the *C. difficile* toxins results from Rho inactivation, subsequent diminishment of RhoK activity, and decreased phosphatidylinositol-bis-phosphate ($PIP_2$) levels as a consequence of reduced phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) activity downstream of Rho and Rac. The actin cytoskeletal effects elicited by LT probably involve inhibition of the Rac-PI4P5K pathway.

ToxA and ToxB alter the barrier function of polarized intestinal cells such as CaCo-2 and T84 by increasing paracellular permeability (Hecht *et al*., 1988; Hecht *et al*., 1992; Johal *et al*., 2004). Concomitant with the permeability increase, ToxA and ToxB disrupt apical and basal actin filaments with a subsequent disorganization of the ultrastructure and component distribution (i.e., ZO-1, ZO-2, occludin, claudin) with TJs, whereas E-cadherin junctions do not evidently undergo any detectable alteration. ToxB decreases actin-ZO-1 association and disperses ZO-1 and occludin from lipid-rich membrane microdomains, without changing the occludin phosphorylation status (Nusrat *et al*., 1995; Chen *et al*., 2002). Since Rho plays an important role in TJ assembly, the effects of ToxB and ToxA on TJs presumably result from Rho glucosylation (Fig. 9.4) (Jou *et al*., 1998). However, ToxA induces protein kinase C (PKC) activation independently of GTPase modification, which could account for altered TJs and increased paracellular permeability (Chen *et al*., 2002). In addition, ToxA induces pro-inflammatory cytokine release, caspase activation, and apoptosis in T84 cells in a Rho-dependent manner, which altogether could contribute to mucosal damage (Brito *et al*., 2002).

*C. difficile* enteritis is characterized by a severe intestinal inflammation, which is toxin-mediated. ToxB and ToxA stimulate release of proinflammatory cytokines from monocytes. Indeed, ToxA increases interleukin (IL)-8 production and causes monocyte necrosis plus mucosal damage through p38MAP kinase activation (He *et al*., 2000). *In vivo*, ToxA is responsible for polymorphonuclear lymphocyte (PMNL) infiltration of the intestinal mucosa, and *in vitro* ToxA causes increased adherence, impaired migration, and reduced oxidative activity among PMNL (Brito *et al*., 2002; Pothoulakis and Lamont, 2001). Additionally, it has been shown recently that T84 cells increase IL-8 production and lose barrier function following ToxA exposure (Johal *et al*., 2004). *In vivo* mouse studies reveal that interferon-gamma and tumor necrosis factor alpha are involved in ToxA effects within the ileum (Ishida *et al*., 2004), while mice given a human intestinal xenograft and subsequently ToxB have an inflammatory response concomitant with a significantly increased expression of IL-8 (Savidge *et al*., 2003).

The LT of *C. sordellii*, which modifies Rac, Ras, Rap, and Ral, also alters permeability of intestinal cell monolayers via redistribution of E-cadherin, but not ZO-1 (Figure 9.4) (Richard *et al*., 1999). ToxB and LT also cause a redistribution of E-cadherin in endothelial cells, probably through Rac inactivation. Additionally, ToxB increases the permeability of both cultured endothelial cells and intact microvessels (Vouret-Craviari, 1999; Adamson *et al*., 2002; Waschke *et al*., 2004).

## TYPE-III VIRULENCE FACTORS WITH GAP ACTIVITY

### *Pseudomonas* exoenzymes

*Pseudomonas aeruginosa* is an opportunistic, nosocomial pathogen that produces several protein toxins called *exoenzymes* (Exo). ExoS, ExoT, ExoU, and ExoY are directly delivered into cells by a type-III secretion system (Table 9.2) (see Chapter 14) (Barbieri, 2000). In contrast, ExoA (~65 kDa) is a classic "A-B," single-chain toxin secreted from the bacterium into the environment and subsequently internalized into a targeted cell via receptor-mediated endocytosis after binding to an alpha-2 macroglobulin/low-density lipoprotein receptor. ExoA ADP-ribosylates elongation factor 2 via a post-translationally modified histidine called *diphthamide*, which subsequently inhibits cell protein synthesis. This intoxication process is similar to that employed by the diphtheria toxin of *Corynebacterium diphtheriae*. As evidenced by crystallography studies and subsequent biochemical analysis, ExoA consists of three domains that include: (i) N-terminal receptor binding region (residues 1–252 plus 365–404); (ii) central translocation region (residues 253–364); and (iii) C-terminal ADP-ribosyl transferase region (residues 405–613) (Pizza *et al*., 1999).

The type-III secreted ExoS molecule (~49 kDa) possesses two characterized activities (Barbieri, 2000). First, ExoS is an ADP-ribosyltransferase that recognizes several unrelated substrates like Ras, IgG2, apolipoprotein A1, as well as vimentin, and it curiously shares more sequence homology with vertebrate ADP-ribosyltransferases than those of procaryotic origin (Ganessan *et al*., 1999). The rather non-discriminating, ADP-ribosyltransferase activity of ExoS is stimulated by an ubiquitous eucaryotic protein (~28 kDa) called *factor-activating exoenzyme S* (FAS), which is a member of the conserved 14-3-3-protein family that is important in cell signaling among eucaryotes (Fu *et al*., 1993). ExoS ADP-ribosylates Ras at two residues, Arg41 and Arg128, that subsequently inhibits Ras-mediated signal transduction (Ganessan *et al*., 1999). The catalytic site on ExoS for ADP-ribosyltransferase activity is located within the C-terminal residues 234–453, with the 420–429 region being important for FAS interactions.

Additionally, ExoS has another activity localized within the N-terminus (residues 1–234), which ultimately disorganizes the actin cytoskeleton and induces cell rounding (Pederson *et al*., 2004). This latter domain of ExoS exerts a noncovalent, GAP activity towards Rho, Rac, and Cdc42 that stimulates the intrinsic GTPase activity of Rho proteins, with a slight preference for Rac. As recently discovered, ExoS activity is exerted in concert with Rho-GDI, which extracts activated Rho-GDP from the membrane and sequesters it in the cytosol (Sun and Barbieri, 2004). Amino acid sequence comparisons reveal that the arginine (Arg) finger, which stabilizes the transition state for GTP hydrolysis and is characteristic of Rho-/Ras-GAPs, is conserved within the N-terminus of ExoS (Ganessan *et al*., 1999). In addition, an Arg146Lys mutation within the Arg finger of ExoS abolishes GAP activity (Goehring *et al*., 1999). This finding further reveals that alteration of the actin filaments by ExoS is mediated by its GAP activity towards Rho, Rac, and Cdc42. The N-terminal domain of ExoS has an almost completely helical structure not related at the amino acid sequence and conformational levels to other Ras-/Rho-GAPs, although its mode of action is the same via insertion of an Arg finger within the catalytic site of Rho proteins (Figure 9.7) (Würtele *et al*., 2001). From a pathogenesis perspective, inactivation of Rho proteins by ExoS could inhibit phagocytosis of *P. aeruginosa* (Goehring *et al*., 1999).

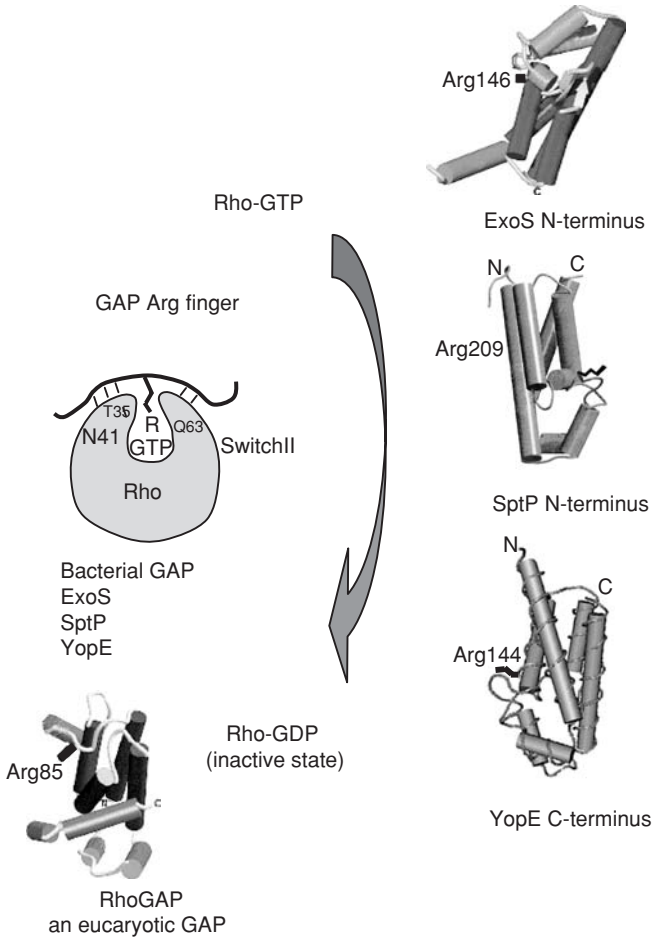



**FIGURE 9.7** Structural comparison of bacterial and eucaryotic GEF proteins, as well as schematic representation of the active Arg finger site.

Like ExoS, the type-III secreted ExoT molecule (~ 50 kDa) is also composed of two domains and shares 76% sequence homology. The N-terminal region of ExoT exerts GAP activity towards Rho, Rac, as well as Cdc42 (Krall *et al*., 2000), and the C-terminus possesses ADP-ribosyltransferase activity; however, this latter activity is only 0.2% of that evident in ExoS. ExoT plays an important role in inhibiting the healing process of damaged lung epithelial cells, and this property is localized within the C-terminus. As proposed for ExoS, the ExoT molecule also prevents phagocytosis by epithelial cells and macrophages (Garrity-Ryan *et al*., 2004).

## *Salmonella* secreted protein tyrosine phosphatase (SptP)

When internalized into cells, *Salmonella* can reverse the actin structures previously activated for bacterial entry, thus enabling the cell to regain its usual shape and intracellular organization. Sequential production by the bacterium, time of delivery into the targeted cell, and/or varying proteasome-dependent degradation patterns within the targeted cell of various virulence factors are probably involved in a coordinated effort upon the actin cytoskeleton that facilitates bacterial invasion and then restoration of the normal cell architecture. SptP represents the main virulence factor involved in the reversion process, and it is delivered into targeted cells by a type-III secretion system (Table 9.2). The N-terminal domain of the molecule is conformationally similar to other type-III secreted virulence factors like *Yersinia* YopE and *Pseudomonas* ExoS, sharing 22–29% amino acid sequence identity (Evdokimov *et al*., 2002). This region of SptP exerts a GAP activity towards Rac and Cdc42, but not Rho. SptP binds to Rac and Cdc42 in the GTP-bound form via SptP residues Thr213 (Switch I and II interface), Gln246 (Switch I interface), as well as Thr249 (Switch I and GDP interface) (Stebbins and Galan, 2000), which ultimately stimulates GTP hydrolysis. As mentioned before, all GAPs possess a conserved Arg that plays a pivotal role in enzyme activity. The Arg finger for SptP has been assigned to Arg209, as cells infected with a *Salmonella* strain producing an Arg209Ala mutant of SptP do not regain a normal actin cytoskeleton after infection. This confirms that SptP reverses the actin cytoskeleton rearrangement by its GAP activity towards Rac and Cdc42 (Fu and Galan, 1999). The C-terminal domain (residues 300–543) of SptP is related to *Yersinia* YopH (30% sequence identity) and several eucaryotic tyrosine phosphatases, with an active site localized to residues 479–489 as well as a phosphate-binding (P) loop motif (Iso/Val)-His-Cys-X-X-Gly-X-Gly-Arg-(Ser/Thr)-Gly typically found in phosphatases (Stebbins and Galan, 2000).

Analysis of a SptP-Rac1 crystal complex reveals that the SptP surface is highly complementary to that of the GTPase. SptP binds Rac exclusively through a four-helix bundle from the N-terminal GAP domain (residues 167–290) that recovers the nucleotide and interacts with both the Switch I (residues 30–41) as well as Switch II (residues 60–71) regions of the GTPase. Helices H3 (residues 207–221; particularly Thr213) and H5 (residues 244–248; particularly Gln246) are the main contact sites with Switch I, while H1 (residues 168–186) as well as H3 target Switch II. The Arg finger (Arg209) is located within helix H3 and inserts deeply into the active site of Rac1. Arg209 interacts with the β-phosphate of GTP and Switch I, thus constraining the Gln61 of Rac1 in a more catalytically favorable conformation for GTP hydrolysis. Although SptP possesses an overall different conformation versus that found in eucaryotic GAPs, its interaction domain with Rho-GTPases mimics that of host GAPs (Figure 9.7) (Stebbins and Galan, 2000). SptP is the smallest, yet one of the more active, Rho family GAPs that possesses unique characteristics involving: (i) location of the catalytic Arg209 on an α-helix, not on a loop, as found in eucaryotic GAPs; and (ii) interactions only with Switch I, Switch II, and the active site of Rac, which differs from eucaryotic GAPs that possess a larger interaction surface. Altogether, this outlines a minimal structure involved in GAP activity and argues for a convergent evolution of eucaryotic and bacterial GAPs. Binding to Rac does not conformationally change the C-terminal domain of SptP, which possesses a tyrosine phosphatase activity that plays an unknown role during intoxication. However, it is possible that the GAP domain targets tyrosine phosphatase to its relevant substrate(s) (Stebbins and Galan, 2000, 2001), which may ultimately down-regulate subsequent nuclear responses to Cdc42 and Rac stimulation (Galan, 2001). Additionally, both tyrosine phosphatase and GAP activities of SptP may reverse MAP kinase activation due to *Salmonella* infection by inhibiting Raf activation, and modifying vimentin involved in forming intermediate filaments as well as membrane ruffles important in bacterial entry (Murli *et al*., 2001; Lin *et al*., 2003).

## *Yersinia* outer protein (Yop) E

*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are Gram-negative, enteric pathogens that cause enteritis, lymphadenitis, extraintestinal abcesses, and septicemia. After ingestion, *Yersinia* penetrate intestinal lymphoid follicles (Peyer's patches) of the terminal ileum, colonize mesenteric lymph nodes, and then

may spread to other organs such as the spleen. Dissemination of *Yersinia* from the intestinal tract begins by entering M-cells via an invasin, a type-III secretion factor that is chromosomally encoded and interacts with β-integrin on the apical surface. Bacteria are then translocated through Peyer's patches and into lymph nodes by way of the M cells, finally multiplying extracellularly since they are resistant to phagocytosis. The main virulence strategy of *Yersinia* involves resistance to polymorphonuclear phagocytosis, which is based on type-III secreted proteins called Yops (Yop-E, -H, -M, -O, -P, and T) with various activities (Table 9.2) (Cornelis, 2002). The genes for Yops are localized on the pYV plasmid (~70 kb) found in *Y. enterocolitica* and *Y. pseudotuberculosis*, as well as an equivalent *Yersinia pestis* plasmid (pCD), and the resultant proteins are responsible for inhibiting phagocytosis, increasing release of proinflammatory cytokines (i.e., IL-12, tumor necrosis factor alpha), and inhibiting nitric oxide production from host cells (Cornelis, 2002; Brubaker, 2003; Monnazzi *et al*., 2004).

YopE (~24 kDa) is one of the most potent *Yersinia* factors responsible for resisting phagocytosis. Expression of GAP-active YopE by *Y. pseudotuberculosis* is an essential virulence factor in infection models with mice and HeLa cells, leading to loss of actin filaments, cell rounding, and inhibited bacterial uptake (Black and Bliska, 2000). YopE shares sequence similarity with the N-terminal domains of *Pseudomonas* ExoS and *Salmonella* SptP. Residues 1–50 of YopE are involved in type-III secretion from the bacterium into macrophages. The crystal structure of YopE GAP domain (residues 90–219) was recently resolved at 2.2 angstrom, thus revealing conformational similarity with ExoS and SptP (Evdokimov *et al*., 2002). In addition, the Arg finger motif is conserved in YopE and an Arg144Ala mutation yields an inactive protein; therefore, YopE acts as a GAP (Figure 9.7) (Black and Bliska, 2000; von Pawel-Rammingen *et al*., 2000). *In vitro* experiments show that YopE stimulates GTPase activity of Rho, Rac, and Cdc42, but not Ras (von Pawel-Rammingen *et al*., 2000). However, in primary human umbilical vein endothelial cells (HUVEC), YopE specifically inhibits Rac-, but not Rho- or Cdc42-, dependent actin structures. In addition, YopE only affects Rac activated via Cdc42 but not direct, Rac-1 induced ruffle formation following stimulation by sphingosine-1-phosphate (Andor *et al*., 2001). Thus, YopE inactivates Rho-GTPases by a GAP activity, thus leading to actin depolymerization and inhibition of phagocytosis. Specific inhibition of the Cdc42-dependent activation of Rac by YopE could block phagocytosis triggered by Fcγ-receptor, since it has been shown that an immunoglobulin receptor-dependent route of internalization is mediated by Cdc42 and Rac, whereas that used by the complement receptor is mediated by Rho (Caron and Hall, 1998).

## OTHER TYPE-III VIRULENCE FACTORS INACTIVATING RHO-GTPASES

### YopT, a cysteine-protease

YopT (~36 kDa) is a type-III cysteine protease that *in vitro* cleaves Rho-GTPases near the C-terminus, thus preventing their association to the membrane normally mediated via a C-terminal prenyl group and yielding irreversibly inactivated Rho-GTPases (Figure 9.8) (Shao *et al*., 2002). YopT disrupts actin filaments and induces cell rounding. When cells are infected with *Y. enterocolitica*, YopT localizes to cell membranes and selectively releases RhoA, but not Rac or Cdc42, from the membrane. RhoA accumulates in the cytosol uncoupled from Rho-GDI and can not activate downstream effectors. In macrophages, YopT disrupts the podosomal adhesion structure and phagocytic cup rich in actin filaments required for bacterial entry (Aepfelbacher *et al*., 2003).

### YopO/YpkA

YopO produced by *Y. enterocolitica* and the homologue YpkA (~80 kDa) from *Y. pseudotuberculosis* are type-III serine/threonine kinases that disrupt the actin cytoskeleton. These effectors directly interact with Rho and Rac independent of their phosphorylation status and GTP or GDP loading state of the GTPases (Table 9.2) (Barz *et al*., 2000; Nejedik *et al*., 2004). The exact mode of inactivation of Rho-GTPases by YopO and YpkA is still unknown.

### YopH

YopH (~50 kDa) is a potent phospho-tyrosine phosphatase that dephosphorylates focal adhesion proteins such as P130 CAS and P125FAK. The N-terminus (residues 1–130) and C-terminus (residues 206–488) are respectively responsible for substrate binding and catalysis (Smith *et al*., 2001).

## TOXINS ACTIVATING RHO-GTPASES

### Toxins that deamidate Rho-GTPases: *Escherichia* CNF and *Bordetella* DNT

The cytotoxic necrotizing factor (CNF) is produced by some pathogenic strains of *Escherichia coli* and consists of two variants, CNF1 and CNF2 (Table 9.1) (see

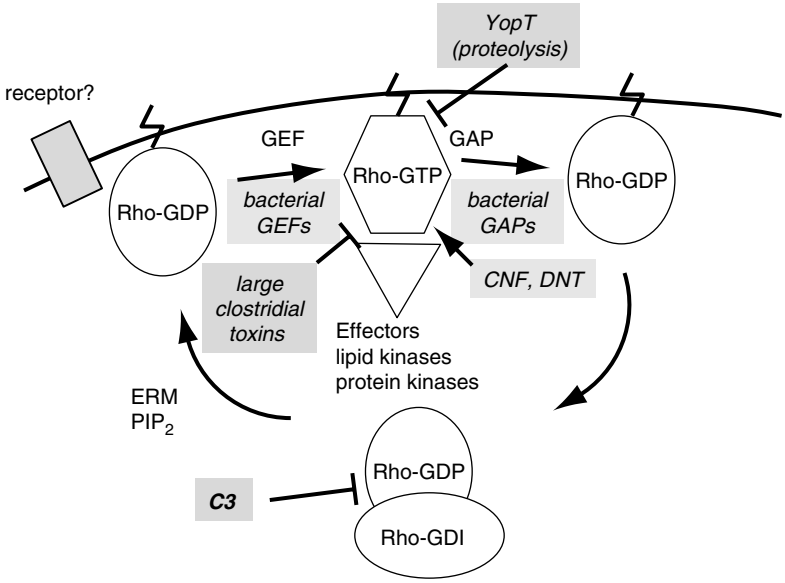



**FIGURE 9.8** Bacterial toxins and virulence factors that target Rho-GTPases. The C3 enzyme and large clostridial toxins enzymatically inactivate Rho-GTPases, whereas CNF/DNT enzymatically activate these targets. Type-III virulence factors from invasive bacteria mimic eucaryotic GEFs or GAPs.

Chapter 11). CNF1 is synthesized by human strains mainly isolated from urinary tract infections and neonatal meningitis, whereas CNF2 is primarily produced by strains of animal origin that are often involved in enteritis and septicemia of calves. Almost all uropathogenic isolates of *E. coli* that produce CNF1 (about 30% of all strains implicated in urinary infections) contain the alpha-hemolysin gene upstream of the *cnf1* gene. The *cnf1* gene is chromosomally located on a pathogenicity island between the alpha hemolysin operon and gene encoding Pap-related and/or S-fimbriae adhesins. The *cnf2* gene is harbored by the Vir plasmid (Bluetow *et al*., 2001).

*E. coli* CNF1 and CNF2 are highly related proteins (86% sequence identity) of ~ 110 kDa, and similar molecules are also produced by other Gram-negative bacilli such as *Bordetella* (i.e., dermonecrotic toxin or DNT; ~159 kDa) (Table 9.1), *Pasteurella* (i.e., *P. multocida* toxin or PMT; ~146 kDa), as well as *Y. pestis* and *Y. pseudotuberculosis* (i.e., cytotoxic necrotizing factor or $CNF_Y$; ~120 kDa) (Bluetow *et al*., 2001; Lockman *et al*., 2002). CNF1, and presumably CNF2, are released from *E. coli* into the local environment, bind to an unknown cell-surface receptor(s), and ultimately enter the cytosol via clathrin-independent endocytosis through late endosomes (Boquet, 2001).

Structurally, the CNF toxins contain three functional regions: (i) a N-terminal domain (amino acids 1–299) involved in binding to a cell surface receptor; (ii) a central domain (amino acids 299 to 720) containing two hydrophobic regions that likely enable translocation of toxin across the cell membrane; and (iii) the C-terminal catalytic domain (residues 720–1014) possessing deamidase activity. The C-terminal domain of CNF1 is unusually folded, since it forms a single compact domain containing a central β-sandwich resulting from two mixed β-sheets surrounded by helices and extensive loop regions. Residues Cys866 and His881, which are essential for catalytic activity, are located in a deep narrow pocket (Figure 9.3). This exclusive positioning of the active site likely dictates substrate specificity and supposes a conformational change for CNF1 and/or Rho to accommodate the switch II region containing the Gln63 target. Cys866 in its thiolate form is responsible for nucleophilic attack on the δ-carboxamide of RhoA Gln63 (Bluetow *et al*., 2001).

The first indication of CNF1 activity towards Rho protein came from an observed electrophoretic shift during SDS-PAGE, which was subsequently confirmed by microsequencing and mass spectrometry. CNF1 preferentially targets Rho and catalyzes deamidation of Gln63 to Glu, but Rac and Cdc42 are also modified at an equivalent residue (Gln61). GDI forms of the Rho-GTPases are not modified by CNF. Gln63 (Gln61) is located within switch II of the Rho-GTPases and has a pivotal role in intrinsic and GAP-stimulated GTPase activity. Deamidation of Gln63 (Gln61) into Glu impairs binding of a water molecule required for GTP hydrolysis (Fiorentini *et al*., 1997; Koch *et al*., 1997); therefore, CNF1 locks Rho-GTPases into a biologically

active form linked to GTP (Figure 9.9). Clearly, CNF2 and *Bordetella* DNT are related to CNF1 structurally, and they also share comparable mechanisms of action. However, DNT/CNF2 act preferentially as transglutaminases, whereas CNF1 is primarily a deamidase (Schmidt *et al*., 1999).

CNF1 and 2 are dermonecrotic upon intradermal injection in rabbits and guinea pigs, as well as lethal by intraperitoneal injection. In cultured cells, CNF1 converts Rho-GTPases into active forms that stimulate downstream signaling pathways leading to multinucleation, increased actin polymerization, reorganization of stress fibers, membrane ruffles, as well as increased phagocytic activity among human epithelial cells. The Rho-Glu63 and Rac-Glu61 forms activate PI4P5K, a subsequent increase of $PIP_2$ levels enhances actin polymerizaton, and activated RhoK stimulates bundling of the actomyosin filaments. The CNF cellular effects vary according to the cell type, which is a consequence of different cytosolic levels of Rho-GTPases. In fibroblasts, such as Vero cells, CNF1 induces formation of dense actin stress fibers and focal contact points, whereas in epithelial cells (Hep2), formation of lamellipodia and filopodia predominate. In both cell types, CNF1 leads to spreading that results from increased formation of actin filaments at the leading edge and anchorage of actomyosin filaments to focal contact points. This suggests that CNF1 activates first Cdc42 and Rac, then Rho, in epithelial cells, whereas the action upon fibroblasts is primarily mediated via Rho (Boquet, 2001). PMT from *P. multocida* also activates Rho and subsequently RhoK leading to actin stress fiber and focal adhesion formation by a yet undefined mechanism. This toxin induces additional cellular effects that include strong stimulation of DNA synthesis, proliferation, and stimulation of phospholipase C (Zywietz *et al*., 2001).

Activation of Rho-GTPases by CNF is only transient, since deamidated Rho-GTPases are ubiquitinated and rapidly degraded by the proteasome. This process favors bacterial internalization by epithelial cells that require Rho-GTPase activation for lamellipodia formation and phagocytosis, followed by down-regulation (Doye *et al*., 2002). In contrast to invasive bacteria, which use distinct activators and inhibitors of Rho-GTPases, CNF alone mediates both sequential activities.

The role of CNF in *E. coli* pathogenesis is still unclear. Various effects of CNF in host cells have been identified, which include stimulation of phagocytosis and bacterial uptake into cells, as well as protection against apoptosis. One of the primary CNF-induced perturbations involves modification of intestinal or urinary tract cell barriers. In intestinal cell monolayers, CNF1 decreases TER and enhances paracellular permeability from the basal to apical surface. Concomitantly, TJ proteins are redistributed in endosomal caveolar compartments, but microvillus F-actin and its binding

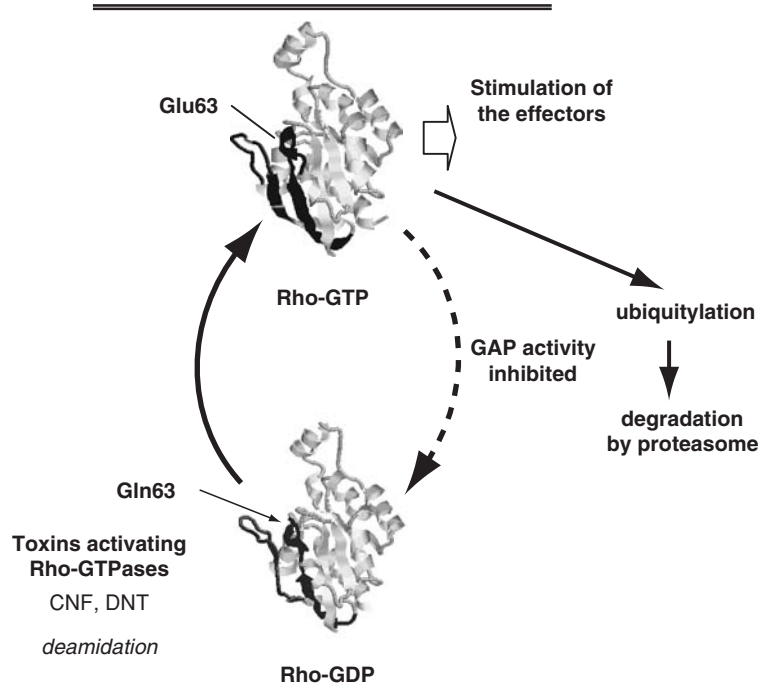



**FIGURE 9.9** Schematic mode of action for Rho-GTPase activating toxins that deamidate Gln63.

protein, villin, disappear. In contrast, AJs containing E-cadherin are preserved during CNF1 intoxication. CNF1-activation of RhoA predominantly mediates the aforementioned cellular effects, although Rac and Cdc42 are seemingly additional targets for CNF1. However, alteration of Rac and Cdc42 may result from an indirect perturbation in the delicate balance of Rho, Rac, and Cdc42 (Hopkins *et al*., 2002).

## VIRULENCE FACTORS WITH GEF ACTIVITY

### *Salmonella* outer protein (Sop) E

*Salmonella* can bind to cells via fimbriae, and then enter by a trigger mechanism consisting of large membrane ruffles that engulf the bacteria. The subsequent actin cytoskeleton and plasma membrane rearrangements are reminiscent of lamellipodia and filopodia induced by growth factors via Rho-GTPases. It was first found that Cdc42 and Rac were involved in *Salmonella*-dependent signaling, which triggers a cell structure rearrangement leading to bacterial internalization. Dominant negative mutants of Cdc42, but less so the Rac variants, inhibit *Salmonella* entry (Chen *et al*., 1996). It was then identified that a type-III secreted protein, called SopE (~25 kDa) interacts with Cdc42 and Rac (Table 9.2) (Hardt *et al*., 1998). Two isoforms exist, SopE and SopE2, which share 70% sequence identity, as well as activate Rho-GTPases like GEFs by catalyzing GDP-GTP exchange. *In vitro* experiments show that SopE exerts a GEF activity towards Rac1, Rac2, Cdc42, RhoG, and to a lesser extent RhoA, but there is no evident activity with RhoB, RhoC, Ran, H-Ras, and TC21 (Hardt *et al*., 1998). In contrast, SopE2 only interacts with Cdc42 and not Rac (Friebel *et al*., 2001). Microinjection of SopE in Cos cells stimulates actin cytoskeleton rearrangement and membrane ruffling similar to that induced by *Salmonella* infection (Hardt *et al*., 1998). SopE binds to switch I and II of Rho-GTPases like other Rho-GEFs, such as the eucaryotic Tiam (T-lymphoma invasion and metastasis)-1 protein (Buchwald *et al*., 2002), ultimately resulting in switch I and II rearrangement and nucleotide release. However, there are noticeable structural differences between SopE and other Rho-GEFs, as the former uses a loop containing the $_{166}$Gly-Ala-Gly-Ala$_{169}$ motif to reorient switches I and II, whereas the catalytic core of other Rho-GEFs consists of an α-helix containing a critical Lys at the active site (Figure 9.10) (Buchwald *et al*., 2002). SopE also acts as a GEF for Rab5, thereby mediating the recruitment of non-prenylated Rab5-GTP into phagosomes containing live *Salmonella*. This promotes phagosome fusion with early endosomes, thus avoiding terminal transport to lysosomes and destruction of the invading *Salmonella* (Mukherjee *et al*., 2001). In addition, activation of Cdc42 and Rac by SopE stimulates JNK and MAP kinase pathways, leading to transcriptional factor activation (Hardt *et al*., 1998). The activity of SopE1 and SopE2 is complemented by another type-III factor, called SopB or SigD, an inositol phosphatase that specifically dephosphorylates inositol (1,3,4,5,6)-pentakisphosphate (Ins1,3,4,5,6P$_5$), producing Ins(1,4,5,6)P$_4$ that acts as an indirect activator of Cdc42 (Zhou *et al*., 2001).

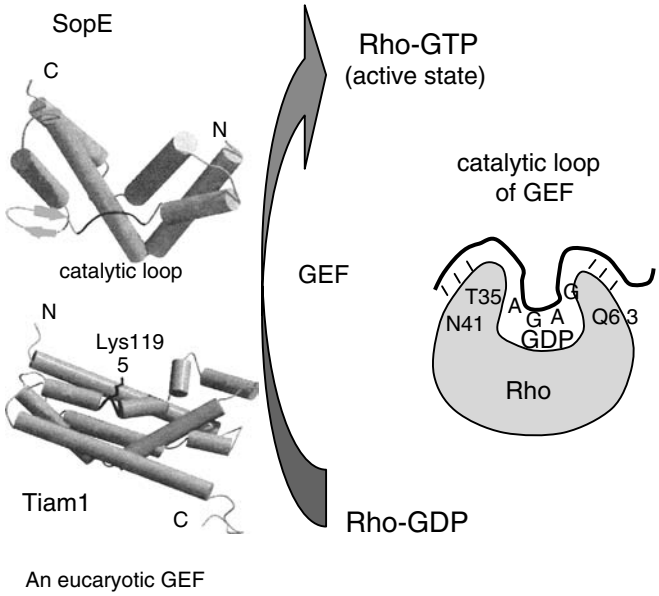



**FIGURE 9.10** Structural comparison of the bacterial GEF SopE (Cdc42/Rac specific) and an eucaryotic equivalent (Tiam1, a Rac GEF), as well as schematic representation of the active site.

## OTHER TOXINS MODIFYING ACTIN POLYMERIZATION

The zonula occludin toxin (ZOT, ~45 kDa) from *V. cholerae* serves a dual function involving phage protein assembly (N-terminus) and enterotoxin activity (C-terminus). The C-terminal part of ZOT is proteolytically cleaved, released, and interacts with a specific receptor that leads to actin polymerization in a PKCα-dependent manner, ZO-1-occludin as well as ZO-1-claudin disengagement, and finally increased cell barrier permeability. The ZOT molecule mimics an endogeneous modulator of TJs and intestinal permeability called zonulin (Wang *et al*., 2000).

*V. cholerae* also produces a RTX (repeats in toxin) toxin containing unusual repeats when compared to

the other RTX toxins. The central domain of cholera RTX cross-links G-actin into dimers, trimers, and higher multimers, thus inducing actin filament depolymerization, as well as subsequent loss of cell barrier function. Depletion of actin monomers, which disrupts the equilibrium between G- and F-actin, probably accounts for the depolymerization of actin filaments (Fullner *et al*., 2000; Fullner and Mekalanos, 2000).

## TOXINS TARGETING INTERCELLULAR JUNCTION MOLECULES

### *Bacteroides fragilis* enterotoxin (see Chapter 28)

*Bacteroides fragilis* enterotoxin (BFT or fragilysin) is a 20 kDa, single-chain protein produced as a 44 kDa protoxin which, upon activation by a serine-type protease, elicits fluid accumulation in ileal loops, as well as causes overt morphological changes in cultured intestinal and renal cells. The toxin is associated with diarrhea in animals and humans. BFT-induced effects on cells include rounding, increased size, and effacement of microvilli as well as apical junction complexes. BFT is a zinc-dependent protease possessing the consensus His-Glu-X-X-His motif, and it cleaves the extracellular domain of E-cadherin, which is primarily found in the zonula adherens that facilitate cell-cell adhesion (Table 9.1 and Figure 9.4). Experimental studies have led to a proposed two-step hypothesis for BFT intoxication, whereby the extracellular domain of E-cadherin is first cleaved by BFT, followed by intracellular degradation via an unidentified protease(s) (Sears, 2001). As a consequence, nuclear signaling and actin rearrangement occurs, which leads to the production of proinflammatory cytokines (Sanfilippo *et al*., 2000), diminished epithelial barrier function, and activation of ion transporters within the apical membrane. The cytotoxic effects of BFT are reversible, as cells adopt a normal morphology 2–3 days after toxin treatment (reviewed in Sears, 2001).

### C. *perfringens* enterotoxin (see Chapter 45)

*C. perfringens* enterotoxin (CPE) is a protein consisting of 319 amino acids (35.3 kDa) and is responsible for a major form of human food poisoning (Table 9.1) (McClane, 2001). It is synthesized by the bacterium during sporulation, lacks a signal peptide, and is released into the environment following sporangium lysis. CPE, like many other *C. perfringens* toxins, is proteolytically activated by serine-type proteases, which probably augment the natural intoxication process within the intestinal tract.

CPE is cytotoxic for Vero cells and intestinal epithelial cells that include CaCo-2, I407, and Hep3b. The first step during intoxication consists of CPE binding, through the 30 C-terminal residues, to membrane protein receptors present in CPE-sensitive cells. The receptors have been identified as claudin isoforms 3, 4, 6, 7, 8, and 14, representing essential components of TJs. CPE binding results in an initial small complex of 90–100 kDa in membranes. At this step, the toxin is largely accessible to antibodies and proteases such as pronase, indicating that it is exposed on the cell surface. Subsequently, a post-binding maturation occurs when CPE-intoxicated cells are incubated at 37°C, which consists of intermediate (135 kDa), and then large (160 or 200 kDa), complexes formed via the addition of other membrane proteins. Development of the large complex is dependent upon CPE residues 45–116, and once formed, it becomes significantly resistant to SDS and pronase treatment, which is likely due to complex insertion into the membrane. Occludin, a major structural protein of TJs, is part of the 200 kDa complex (Singh *et al*., 2000). The CPE large complex increases membrane permeability to small molecules (< 200 Da), possibly by pore formation induced by the 160 kDa complex. The loss of various small molecules (i.e., ions, amino acids, nucleotides, etc.) ultimately inhibits macromolecule synthesis and induces cytopathic effects leading to morphological changes, altered permeability for large molecules, and finally cell lysis. Additionally, the 200 kDa complex formed in polarized CaCo-2 cells removes occludin from TJs and increases paracellular permeability (Figure 9.4) (McClane, 2001; Singh *et al*., 2001). This mechanism for CPE intoxication is probably responsible for the intestinal fluid accumulation and epithelium desquamation observed *in vivo*. In the small intestine of experimental animals, CPE induces a desquamation particularly localized at the villi tips with a rapid loss of fluid and electrolytes. The ileum is the most CPE-sensitive segment of the intestinal tract, whereas little or no effects are in the colon. The histopathological changes elicited by CPE probably play a major role in the fluid and electrolyte perturbations (McClane and Rood, 2001).

## VIRULENCE FACTORS PROMOTING ACTIN NUCLEATION AND POLYMERIZATION

### *Listeria* ActA and *Shigella* IcsA

*Listeria monocytogenes, Shigella dysenteriae,* and *Rickettsia species* are intracellular pathogens that escape phagosomal

endosomes and move throughout the cytosol by using the actin machinery provided by the host's cell. Actin polymerization at one pole of the bacterium ensures intra-/inter-cellular movement, which is responsible for bacterial spreading throughout host tissues. *L. monocytogenes* and *S. flexneri* respectively synthesize surface proteins, ActA and IcsA, which are polar-anchored into the outer bacterial membrane and induce formation of a polarized actin tail that propels the bacterium (Table 9.3) (Cossart and Sansonetti, 2004).

ActA is a 610 amino acid protein consisting of a C-terminus (residues 395–584) anchored in a polar fashion to the outer bacterial membrane, a central domain containing four proline-rich repeats (residues 234–394), and an N-terminal part (residues 1–233) with two regions essential for actin polymerization. An acidic domain (residues 33–63) and residues ($Lys_{117}$-Lys-Arg-Arg-$Lys_{121}$) called the *T,* or tail, region play a key role in actin nucleation that likely occurs by binding and activating the Arp2/3 complex (Welch *et al*., 1998). The T region shares sequence homology with an active domain from the Wiskott-Aldrich-Syndrome protein (WASP) family, which contains WASP expressed exclusively in hematopoietic cells, and the most ubiquitous N-WASP especially enriched in the brain (Higgs and Pollard, 2001). The proline-rich domain, which binds the vasodilator-stimulated phosphoprotein (VASP) and subsequently profilin, has an accessory role in actin polymerization that dictates speed and direction of bacterial movement, as well as virulence (Auerbuch *et al*., 2003). VASP clearly increases the velocity of *Listeria* motility. VASP associated with ActA immobilized on bacteria or beads reduces the branching of actin filaments, and thus probably enhances bacterial motility by properly orienting actin filaments at the bacterial surface (Samarin *et al*., 2003; Sechi and Wehland, 2004). The mode of action of VASP is complex, as it enhances Arp2/3 complex-mediated nucleation of actin filaments, antagonizes the binding of capping proteins to barbed ends probably by an indirect competition facilitating filament elongation, and VASP also regulates actin filament branching (Sechi and Wehland, 2004).

ActA mimics N-WASP in nucleating actin monomers and promoting its polymerization into "comet-like" actin filaments via the Arp2/3 complex (Figure 9.11). WASP, N-WASP, and the related WAVE (or Scar) proteins contain a VCA domain (verprolin-homology (V), cofilin-homology (C) (Lys-Arg-Ser-Lys-Ala), and acidic consensus sequence (A) (Asp-Glu-Trp-Glu-Asp)) within the C-terminus, which is required for actin polymerization through Arp2/3 activation. The V, also known as the WASP homology 2 (WH2), domain is a G-actin binding site, while the CA domain recruits and activates Arp2/3 complex. Other regions of these molecules are involved in regulating VCA activity. WASP and N-WASP possess a N-terminal WH1 domain that binds WASP interacting protein (WIP), a basic region that binds $PIP_2$, a Cdc42-specific CRIB motif, and a proline-rich central region that recruits SH3 domains from various proteins that include profilin. The N- and C-termini of WASP naturally lead to an auto-inhibited conformation (Higgs and Pollard, 2001; Takenawa and Miki, 2001).

Cdc42-GTP, but not Cdc42-GDP, competes with the C-terminus for binding to the N-terminus; therefore, inducing a conformational change that enables the

**TABLE 9.3** Bacterial/viral surface factors promoting actin nucleation and polymerization involved in intra-/inter-cellular motility or actin pedestal formation

| Effectors | Pathogens | Cellular targets | Effects | Ref |
|---|---|---|---|---|
| ActA | *Listeria monocytogenes* | Arp2/3 | intra-/inter-cellular motility | (Cossart and Sansonetti, 2004) |
| IcsA | *Shigella flexneri* | N-Wasp | intra-/inter-cellular motility | (Cossart and Sansonetti, 2004) |
| RickA | *Rickettsia coronii* | Arp2/3 | intra-/inter-cellular motility | (Gouin *et al*., 2004) |
| A36R | *Vaccinia virus* | N-WASP | intra-/inter-cellular motility | (Moreau *et al*., 2000) |
| Y144R | *Yaba-like disease virus* | unknown | intra-/inter-cellular motility | (Law *et al*., 2004) |
| Internalin A | *Listeria monocytogenes* | E-cadherin | actin polymerization in a Rac and Arp2/3-dependent manner | (Cossart and Sansonetti, 2004) |
| Internalin B | *Listeria monocytogenes* | Met transmembrane receptor tyrosine kinase | actin polymerization | (Cossart and Sansonetti, 2004) |
| Invasin | *Yersinia* | β1-integrin | actin polymerizaztion in a FAK, Src, and Rac-dependent manner | (Cossart and Sansonetti, 2004) |
| Pilus type IV Opa, Opc | *Neisseria* | CD46 | Rho activation | (Plant and Jonsson, 2003) |

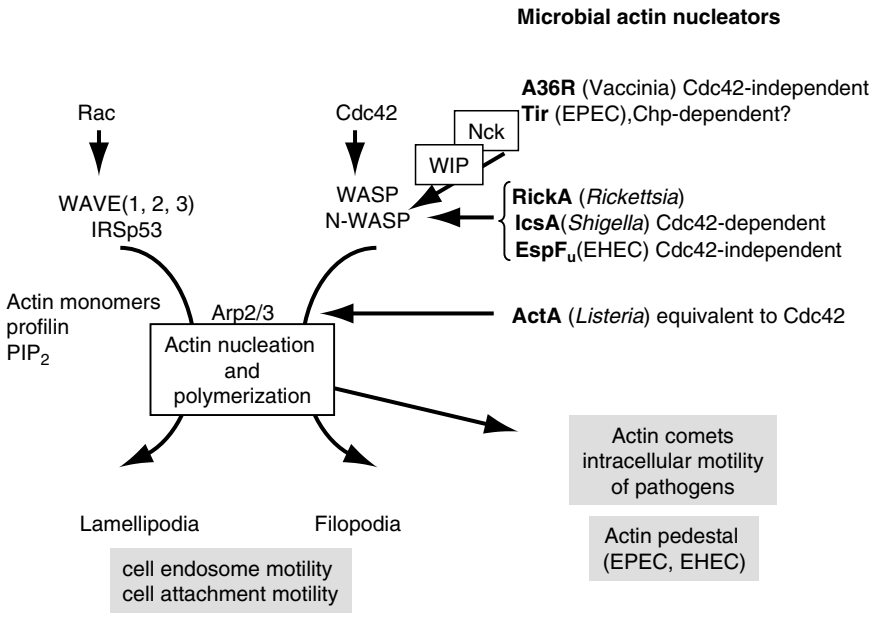



**FIGURE 9.11** The main eucaryotic and microbial virulence factors involved in actin nucleation.

VCA region to bind and activate the Arp2/3 complex, as well as facilitate interactions between actin monomers and profilin. The Arp2/3 complex recruits actin monomers and provides a nucleation site for actin polymerization as well as growth of nascent F-actin (Bishop and Hall, 2000; Carlier *et al*., 2003).

Like *Listeria,* the motility model for intracellular *Shigella* also involves a critical surface protein, IcsA, which promotes nucleation and polymerization of actin. IcsA is 120 kDa and has no sequence similarity with *Listeria* ActA (Cossart and Sansonetti, 2004). The molecule is inserted into the outer bacterial membrane through its C-terminus (37 kDa sized fragment) in a unipolar fashion, and binds to N-WASP, via glycine-rich repeats within the N-terminus (Sansonetti, 2002). IcsA mimics the eucaryotic Cdc42 by binding and activating N-WASP, which then recruits the Arp2/3 complex (Figure 9.11). This ternary structure triggers actin nucleation and filament elongation, which ultimately promotes bacterial movement throughout the cytoplasm of a host cell (Egile *et al*., 1999). IcsA also binds to vinculin, which interacts with VASP through a proline-rich central motif, which in turn recruits profilin via another proline-rich, repeat region. At the fast-growing end of actin filaments, profilin recruits actin monomers and $PIP_2$, thus enhancing actin polymerization (Loisel *et al*., 1999). However, although profilin is required for efficient *Shigella* motility and a vinculin-VASP complex is formed on intracellular *Shigella*, the recruitment of profilin seems independent of VASP (Ally *et al*., 2004). Additionally, Cdc42 facilitates the initiation of tail formation, but is seemingly dispensable thereafter (Suzuki *et al*., 2000).

*Rickettsia conorii*, which is responsible for tick/flea-borne spotted fever, also produces a surface protein called RickA (517 amino acids) that recruits and activates the Arp2/3 complex, thus inducing actin nucleation and subsequent polymerization (Figure 9.11) (Gouin *et al*., 2004). The activation process of Arp2/3 by RickA, although less efficient than that induced by *Listeria* ActA, leads to filopodia formation and bacterial locomotion.

Finally, it is noteworthy that many viruses also manipulate the host actin cytoskeleton to their advantage, such as vaccinia, which induces actin tail formation for intra-/inter-cellular motility (Cudmore *et al*., 1997). The cascade of events triggered by vaccinia virus seems to involve the following sequence: (i) the viral coat protein A36R is activated by a host Src kinase; (ii) recruitment of the adaptor protein Nck; (iii) binding of Nck to WIP-N-WASP complexes; (iv) and subsequent association with the Arp2/3 complex in a Cdc42-independent manner (Figure 9.11) (Frischknecht *et al*., 1999; Moreau *et al*., 2000).

## *Salmonella* invasion protein (Sip)A and SipC

Reorganization of the actin cytoskeleton involved in cell invasion by *Salmonella* requires an additional type-III secreted factor called SipA (Table 9.2) (Lilic

*et al*., 2003). *Salmonella* triggers rearrangement of the actin cytoskeleton and membrane ruffling at the bacterium-cell contact site, thus facilitating bacterial entry by means of several factors (Hayward and Koronakis, 2002). SipA (~74 kDa) molecularly mimics eucaryotic nebulin (600–900 kDa) by sharing common binding sites on actin, which are not evident on the procaryote equivalent MreB, that cross-link and ultimately stabilize F-actin strands (Galkin *et al*., 2002). Experiments show that a *Salmonella* mutant defective in SipA, but possessing other factors that stimulate actin polymerization, (i.e., SopE) induces a diffuse rearrangement of the actin cytoskeleton and is impaired in cell entry (Higashide *et al*., 2002). By competing with gelsolin and ADF/cofilin for binding sites on F-actin, SipA and specifically the C-terminal 238 residues can reduce concentrations of actin monomers below a critical level required for actin polymerization (Zhou *et al*., 1999). This action ultimately leads to increased stability of actin filaments and bacterial entry (Galan and Zhou, 2000). Additionally, SipA binds to fimbrin (or T-plastin), an actin binding protein that packages actin into very tight parallel bundles, which markedly increases F-actin stability (Galan and Zhou, 2000). Fimbrin is mainly localized to membrane ruffles and filopodia following Cdc42 activation. SipA probably acts in concert with SopE, which initially recruits actin filaments and fimbrin into membrane ruffles through Cdc42 activation. Subsequently, SipA reinforces and stabilizes actin filaments leading to a more efficient uptake of *Salmonella* (Zhou *et al*., 1999).

SipC (~45 kDa) is another secreted *Salmonella* type-III protein that directly nucleates and polymerizes actin, as well as cross-links F-actin strands, independent of cellular factors like Rho-GTPases; however, SipA stimulates SipC activity via an unknown mechanism. The N- and C-domains of SipC respectively provide F-actin bundling and actin-nucleating activity. Although SipC also associates with SipB, and this complex binds to lipid membranes, the precise role played by SipC is still elusive. The SipC-SipB complex could target cell membranes at the contact site with the bacterium, perhaps promoting actin condensation into filaments under the membrane of an invading *Salmonella* (Hayward and Koronakis, 1999).

## *Shigella* invasion plasmid antigen (Ipa)C

Invasion and colonization of the colonic epithelium by *Shigella* is a prerequisite step of shigellosis, which represents a severe form of diarrhea that can be fatal and is most commonly experienced by children in developing nations (Jennison and Verma, 2004). Like *Salmonella*, *Shigella* also induces at the contact point with a cell the formation of membrane extensions that raise and engulf the bacterium, thus facilitating internalization. As with many other Gram-negative pathogens, *Shigella* entry into cells depends upon injection of virulence factors through a type-III secretion apparatus (Tran Van Nhieu *et al*., 2000). Among the various virulence factors produced by *Shigella*, IpaC (~45 kDa) plays a key role in filopodia formation and is biochemically similar, but not identical, to *Salmonella* SipC. It was first found that *Shigella* entry requires an actin cytoskeleton rearrangement mediated by Cdc42 and Rac, while ezrin is recruited to the entry site by Rho (Tran Van Nhieu *et al*., 1999). Purified IpaC, when added to semi-permeabilized cells or microinjected into intact cells, promotes the formation of lamellipodia and filopodia. The C-terminal region of IpaC activates actin polymerization, which is a process inhibited by IpaC-specific monoclonal antibodies (Tran Van Nhieu *et al*., 1999). A central, hydrophobic, and highly immunogenic region is important for phospholipid interactions, while the N-terminus facilitates secretion from the bacterium as well as conversion of filopodia into lamellipodia. IpaC evidently interacts with Cdc42 by a novel mechanism, or acts upstream of the Rho-GTPases, possibly through Src kinases. It has been shown that Src tyrosine kinase activity is necessary for activating Cdc42 and Rac, but down-regulates Rho, during *Shigella* entry into cells (Tran Van Nhieu *et al*., 2000; Dumenil *et al*., 2000).

## Tyrosine kinase signaling and actin reorganization (enteropathogenic *E. coli*)

Various members of the Enterobacteriaceae family, such as the enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Citrobacter rodentium*, use the actin cytoskeleton machinery to specifically alter the intestinal mucosa via attaching/effacing lesions that are associated with disease pathology. These bacteria first adhere to the enterocyte membrane, transducing signals that lead to microvilli effacement and formation of an actin-rich bacterial adhesion structure, known as a "pedestal." EPEC and EHEC inject a 75 kDa, type-III-secreted Tir (translocated intimin receptor) that inserts into the host cell membrane and subsequently functions as a receptor for the bacterial, surface-exposed intimin (~97 kDa) (Table 9.2). The N- and C-termini of membrane-localized Tir are intracellular, but the intimin binding domain (residues 272–362) is extracellular and specifically interacts with the 181 C-terminal residues of intimin. In EPEC, Tir is phosphorylated at Tyr474 and binds to the adaptor protein Nck, which recruits and activates N-WASP as well as the Arp2/3 complex (Figure 9.11). This promotes

actin nucleation and polymerization required for pedestal formation. In contrast to EPEC, the EHEC Tir is not phosphorylated, thus suggesting that actin polymerization in the pedestal implicates another bacterial effector recently identified as *E. coli* secreted protein (Esp) $F_U$ (Campellone and Leong, 2003). Use of $EspF_U$ by EHEC strains circumvents the need for Tir phosphorylation and Nck, which are necessary for actin polymerization by EPEC strains. The association of EHEC Tir and $EspF_U$ leads to recruitment of N-WASP and actin assembly (Campellone *et al*., 2004). Tir-mediated polymerization of actin is independent of Cdc42 GTPase, but possibly dependent upon a Cdc42 homologue called Chp-GTPase, which is in contrast to the cytoskeletal effects induced by another EPEC effector known as the mitochondrial-associated protein (Map) (Kalman *et al*., 1999; Kenny *et al*., 2002). The EPEC Map has two distinct functions that include mitochondrial dysfunction involving loss of membrane potential and formation of Cdc42-dependent filopodia (Kenny *et al*., 2002). A combination of the Tir-induced signaling pathway that antagonizes filopodia formation, perhaps via GAP activity towards Cdc42, and signals triggered by Map can mediate a complex, coordinated bacterial invasion of epithelial cells (Jepson *et al*., 2003).

## BACTERIAL REORGANIZATION OF ACTIN AND PHYSIOPATHOLOGICAL EFFECTS

### Alteration of cell barriers

The first line of defense for any organism against infection is an intact epithelial cell barrier forming the skin and various mucosal surfaces of the respiratory, digestive, and uro-genital tracts, which also selectively controls passage of essential molecules with the external environment. The endothelia represent other internal cell barriers regulating solute exchange and leucocyte trafficking between blood and tissues. Over time, bacterial pathogens have developed different strategies to counteract these barriers.

Barrier cell function is mediated by intercellular junctions, which in epithelia are comprised of TJs, AJs, and desmosomes, while the endothelial equivalents include TJs, AJs, and gap junctions. In epithelia, intercellular junctions are well organized with TJs located at the apical surface and AJs along the basolateral surface between two adjacent cells. AJs play an important role in endothelia, whereas TJ organization varies along the vascular tree. Although TJs are well developed in endothelia of brain and large arteries, they are poorly organized in peripheral vessels such as venules.

TJs are protein complexes organized into parallel strands and contain integral transmembrane components, such as occludin, claudins, junctional adhesion molecules (JAM), as well as TJ plague proteins (i.e., ZO-1, ZO-2, ZO-3, cingulin). These proteins cross-link two adjacent cells by connecting integral TJ proteins to the actomyosin ring, as well as recruit cytosolic signaling molecules and many regulatory proteins. AJs consist of transmembrane cadherins, mainly vascular endothelium (VE)-cadherin, which form homophilic interactions within the intercellular space and are linked into the cytosol to actin filaments through α-, β-, and γ-catenins. The actin cytoskeleton and Rho-GTPases are important regulators of TJ and AJ function, which are controlled by a wide range of growth factors, cytokines, drugs, and hormones (Wojciak-Stothard and Ridley, 2003; Bazzoni and Dejana, 2004; Harhaj and Antonetti, 2004; Schneeberger and Lynch, 2004).

A common means of bacterial entry into an organism involves the digestive route. Many bacteria use the actin cytoskeleton to counteract the barrier function provided by the intestinal epithelium. Toxin secretion allows bacteria with neither type-III secretion nor invasive mechanisms to alter the intestinal barrier, gain access to essential nutrients, and invade internal tissues (Figure 9.1). Thereby, secreted toxins act at a distance from the infection site and produce severe as well as irreversible alteration of cell barriers. For example, clostridia have no adherent or invasive properties and only multiply in the intestinal content if the resident flora do not exert adverse effects. Only toxigenic strains ultimately interact with the intestinal mucosa and are responsible for gastro-intestinal diseases and enterotoxemia. Clostridia producing actin-ADP-ribosylating toxins cause a complete loss of actin filaments, cell morphology changes, and drastic alteration of TJs and AJs (Figure 9.4). Although a finely-tuned cooperation between Rho, Rac, and Cdc42 is required for cell barrier function, Rho has an important role in regulating TJ integrity mainly by controlling the apical actin ring contractility via RhoK (Wojciak-Stothard and Ridley, 2003). The toxins inactivating or activating Rho, but not other Rho-GTPases, alter epithelial barriers as well as cause necrotic lesions, and include: (i) *C. difficile* ToxA and ToxB involvement in pseudomembranous colitis; (ii) *C. sordellii* HT association with hemorrhagic enteritis; and (iii) *E. coli* CNFs role in hemolytic uremic syndrome (Figure 9.8). It is also the case for bacteria acting on the epithelium of the epidermis, like EDIN-producing *S. aureus* and skin suppurations (see above). Interestingly, Rho-GTPase-inactivating toxins from

clostridia involved in gangrene (i.e., *C. sordellii* LT, *C. novyi* α-toxin) mainly target Rac, and their preponderant effect upon the host is extensive edema. Gangrenous clostridia enter soft tissues via a tegument break, like a deep-penetrative wound, and upon growth in an anoxic environment these bacteria secrete toxins that act on endothelial cells and modify cell barrier permeability, where Rac-dependent AJs are predominant. Toxins like LT also cause muscle cell necrosis (Barbier *et al*., 2004), but additional necrotic toxins or extracellular hydrolytic enzymes (i.e., neuraminidases, phospholipases, proteases) are also produced by these bacteria. Moreover, toxins disrupting actin filaments via direct interaction with actin monomers (i.e., clostridial actin-ADP-ribosylating toxins, *V. cholerae* RTX) or increasing actin polymerization (*V. cholerae* ZOT) alter intercellular junctions and cell barrier permeability (Figure 9.4).

Another mechanism for altering cell barrier function consists of a direct toxin interaction with intercellular junction components. CPE binds and removes claudin-3 and -4, resulting in disintegration of TJ strands and increased permeability. BFT has a proteolytic activity towards E-cadherin. A *V. cholerae* hemagglutinin/protease (HA/P) specifically degrades occludin, resulting in the breakdown of TJ function (Wu *et al*., 2000). Summarized modes of action of bacterial toxins interfering with cell barrier function are shown in Figure 9.4.

## Cell invasion and transmigration through cell barriers

Other pathogens undergo more complex interactions with epithelial or endothelial cells that include hijacking the regulation of actin polymerization through bacterial surface proteins or effectors directly injected into target cells. Instead of secreting toxins that diffuse and indiscriminately disrupt all the cells from a monolayer, these pathogens restrict their activity to the cells to which they adhere. By this way, invasive bacteria induce their phagocytosis into normally non-phagocytic cells, and other bacteria remain adherent to the membrane leading to intimate interactions with a host cell.

Invasive bacteria escape host defenses once inside epithelial or endothelial cells, by two main types of entry mechanisms. The trigger mechanism used by *Salmonella* and *Shigella* consists of bacteria-cell contact through a type-III system, injection of virulence factors into the cytosol, drastic rearrangement of the cortical actin cytoskeleton, formation of ruffles and a phagocytic cup, internalization of bacteria into vacuoles, and subsequent restoration of the normal actin architecture (Table 9.2). *Salmonella* entry, the best characterized to date for any microorganism, is based on type-III-effectors like SopE and SptPs which respectively mimic eucaryotic GEFs and GAPs to manipulate the actin machinery. Although procaryotic GEFs and GAPs share no significant relatedness at the structural level with their eucaryotic counterparts, they retain a similar enzymatic site, suggesting a convergent evolution between pathogen and host. Additional type-III factors, like ADP-ribosylating proteins (SpvB) and an actin nucleator (SipC), are also involved in *Salmonella* pathogenensis.

*Shigella* uses a different set of type-III effectors, which also successively trigger actin polymerization (IpaC) and depolymerization (IpaA) via a more complex signaling pathway than regulating the Rho-GTPases cycle. IpaA binds to vinculin, but its mode of action remains unknown. *Shigella* enters the intestinal mucosa through M cells, which then initiates an inflammatory response, transmigration of neutrophils, opening of TJs, and enhances access to the basolateral surface of epithelial cells where the bacteria bind to integrins via IpaB/C.

In contrast, *Listeria* and *Yersinia* enter epithelial cells by a zipper mechanism, respectively mediated by the bacterial membrane proteins, invasin and internalin, which interact with cell surface receptors and activate a signaling cascade leading to a moderate rearrangement of the actin cytoskeleton for bacterial internalization (Table 9.3). Although this process is incompletely understood, it does involve Rac and Arp2/3 (Cossart and Sansonetti, 2004). *Neisseria* also uses a zipper mechanism to enter epithelial and endothelial cells triggered by adhesion mediated by type IV pili, bacterial surface proteins (i.e., Opa, Opc), and host cell receptor (CD46), which ultimately leads to activation of Src family tyrosine kinase (Table 9.3). In endothelial cells, type IV pilus-mediated adhesion is associated with recruitment of ERM proteins, activation of Rho and Cdc42, as well as actin rearrangement leading to microvilli formation, bacterial internalization, and subsequent bacterial transcytosis (reviewed in Nassif *et al*., 2002; Plant and Jonsson, 2003).

Unlike many of the other type-III transmitted effector molecules described in this chapter, CNF1 produced by uropathogenic *E. coli* enters epithelial cells by a mechanism different than the type-III secretion system. For ultimately enhancing phagocytosis, CNF1 ensures the transient stimulation of actin polymerization that leads to the formation of phagocytic vesicles, followed by down-regulation of the actin cytoskeleton (see above). CNF1 also causes a redistribution of TJ molecules and alters epithelium permeability (Hopkins *et al*., 2002). Although CNF1 activates Rho-GTPases in contrast to *C. difficile* ToxA and ToxB, both types of toxins increase the cell barrier permeability,

and this information suggests that a finely-tuned balance is required for cell barrier function. *Pasteurella* PMT, which is related to CNF1, also increases endothelial permeability by activating Rho, RhoK, and myosin light chain phosphorylation by an undefined pathway (Essler *et al*., 1998). Other pathogens intimately interact with the epithelial cell surface without invading cells, but their survival depends upon modifying the actin cytoskeleton and altering cell barrier integrity.

*Helicobacter pylori* adheres to gastric epithelial cells and injects a cytotoxic-associated antigen (CagA) protein through a type IV secretion system (Table 9.2). CagA is phosphorylated by a Src family kinase and binds to ZO-1 as well as JAM, leading to disruption of epithelial barrier function via a not yet defined mechanism (Amieva *et al*., 2003). Another unidentified type IV effector activates Rac as well as Cdc42, thus increasing epithelial cell motility and contributing to gastric carcinogenesis (Naumann and Crabtree, 2004).

EPEC and EHEC also intimately attach to intestinal cells, thus inducing loss of microvilli and generating attaching-effacing lesions, but these bacteria are poor invaders of host cells (see above). In addition, these entero-pathogens alter the intestinal barrier function. EPEC infection causes type-III-mediated phosphorylation of myosin light chain kinase, resulting in increased contraction of the actomyosin ring, as well as dephosphorylation and redistribution of occludin (Simonovic *et al*., 2000). In EHEC, the type-III-Esp$F_U$ that is not involved in attaching-effacing lesions can facilitate redistribution of occludin and increased cell barrier permeability (Table 9.2) (McNamara *et al*., 2001).

Finally, various *Chlamydia* species represent obligatory intracellular procaryotes that possess a distinct, biphasic development cycle. As pathogens, they are quite successful and cause prevalent forms of sexually transmitted disease as well as blindness throughout the world. These microorganisms employ a type-III secretion system to gain entry into cells, following the binding of elementary bodies to eucaryotic surfaces via lipid rafts (Fields *et al*., 2003; Stuart *et al*., 2003). Overall, little is known about chlamydial type-III secretion and effector molecules relative to other microorganisms; however, activation of Cdc42 and Rac are important for the infection process, unlike Rho (Subtil *et al*., 2004). Recently, the CT456 molecule has been identified as a type-III secreted effector that recruits actin to the elementary body-cell contact site for uptake (Table 9.2) (Clifton *et al*., 2004).

## Antiphagocytosis

When pathogens overcome the body's primary line of defense, represented by the tegument or epithelial barrier, they encounter macrophages, dendritic cells, and polynuclear lymphocytes that recruit additional cellular help towards the infection site via cytokines and chemokines. Additionally, stimulation of host innate immunity occurs through pattern recognition receptors on professional phagocytes that target specific pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide, lipoteichoic acid, and CpG motifs. To overcome this swarming, inflammatory approach for protecting the host, some bacteria have developed strategies that prevent phagocytosis based upon manipulating the host's actin cytoskeleton (Figure 9.11). The best characterized examples of microorganisms that prevent phagocytosis have been derived from studies with *P. aeruginosa* and *Yersinia* species.

*P. aeruginosa* is a common environmental bacterium with opportunistic, pathogenic tendencies. This bacterium normally exploits an immunocompromised host, subsequently causing various maladies linked to the skin of burn patients, lungs (cystic fibrosis), urinary tract, surgical wounds, and ulcerative keratitis. Virulence factors associated with *P. aeruginosa* infections include antibiotic resistance, pili, flagella, and elastase, which all contribute in different ways to successful invasion as well as dissemination. In addition, during an infection, this microorganism secretes various type-III effectors, which play different roles that include the inhibition of phagocytosis and wound healing. In particular, *Pseudomonas* ExoS down-regulates actin polymerization in macrophages through a GAP activity towards Rho-GTPases and ADP-ribosylation of other targets.

Foodborne pathogens like *Y. enterocolitica* and *Y. pseudotuberculosis* can cross the intestinal epithelium through M cells, thus reaching the lymphoid tissues within Peyer's patches. This process induces a recruitment of phagocytic cells that includes polymorphonuclear leucocytes and monocytes with subsequent formation of microabcesses. The host inflammatory response does restrict *Yersinia* invasion; however, this bacterium can resist phagocytosis and disseminates to the mesenteric lymph nodes, liver, and spleen. When passed through M cells, *Yersinia* produces various virulence factors that afford a microbial defense towards innate immunity and subsequent extracellular multiplication in lymphoid tissues. Among the important virulence factors, *Yersinia* secretes a set of type-III proteins (i.e., YopE, YopH, YopT, and YpkA/YopO) that have a negative impact upon cytoskeletal dynamics and resist phagocytosis. YopE, YopT, and YpkA/YopO act on Rho-GTPases via different mechanisms. YopE has GAP activity, while YopT also inactivates the same targets via cysteine protease activity. YpkA/YopO

binds to actin and Rho-GTPases, but its mode of action is still unclear; whereas YopH is a phospho-tyrosine phosphatase acting on the focal adhesion protein P130 (Cornelis, 2002). It is noteworthy that for a more efficient antiphagocytic effect, these bacteria down-regulate the actin cytoskeleton by simultaneously inactivating pivotal targets necessary for actin polymerization along with several effectors possessing different mechanisms of action.

### Intracellular motility

Another strategy for pathogen escape from host defenses consists of intracellular replication, movement, avoidance of degradation mechanisms (i.e., lysosomes), as well as eluding engulfment by professional phagocytes. *Salmonella* survive in macrophages through type-III effectors that enable survival in an intracellular compartment sheltered from the NADPH oxidase-dependent respiratory burst and ensuing oxygen radicals. Other intracellular bacteria, like *Listeria*, can lyse the intracellular vacuole membranes through a cholesterol-dependent, pore-forming toxin called listeriolysin O, phosphatidylinositol-phospholipase C, and phosphatidycholine-phospholipase C; whereas, IpaA molecule serves the same function for *Shigella*. The bacteria can then replicate in the cytosol, thus avoiding trafficking/destruction to the lysosomes. To colonize host tissues, these pathogens use actin dynamics similar to the processes occurring at the leading edge of a moving eucaryotic cell, thus propelling themselves within the cytosol and into adjacent cells. It is interesting to note that *Shigella*, which are strictly adapted to humans and monkeys, as well as an environmental opportunistic pathogen, like *Listeria*, both exploit related actin-based cellular mechanisms to enter cells and disseminate intracellularly. The *Rickettsia* and vaccinia virus also possess a similar mode of intracellular, actin-based motility. Certain bacterial or viral membrane proteins, which mimic a eucaryotic counterpart, play a key role in triggering host actin nucleation as well as polymerization at one pole of the pathogen and include: *Listeria* ActA, *Shigella* IcsA, *Rickettsia* RickA, and vaccinia virus A36R (Table 9.3 and Figure 9.11).

## CONCLUSION

Actin supports numerous fundamental processes needed for eucaryotic life, and many actin-based mechanisms are involved in procaryotic interactions with eucaryotic cells, which thus forms the basis of various bacterial and viral infections/intoxications. From the pathogen's perspective, an ability to manipulate the host is desirable for obtaining additional nutrients, avoiding the immune system, and ultimately disseminating to a new host. Relative to eucaryotes, many procaryotes contain at most a "rudimentary" form of an actin cytoskeleton, but various microbial pathogens have cleverly evolved ways of exploiting the host actin machinery for their own objectives by producing toxins or virulence factors that interfere with actin regulation. Among the numerous partners responsible for actin regulation, procaryotic toxins and virulence factors act at two levels that involve actin monomers and Rho-GTPases. Various toxins enzymatically modify actin monomers that prevent further polymerization, while in contrast several procaryotic surface proteins act as nucleating factors permitting intra-/inter-cellular motility of pathogens. Rho-GTPases also represent either a direct target of various toxins and type-III secreted factors or an indirect target regulated via GEFs as well as GAPs and modulated by equivalent procaryotic factors. Rho-GTPases play a key role in regulating actin polymerization, since they amplify signals through an effector cascade and then direct them towards a particular pool of actin that facilitates a coordinated cell function like endocytosis/phagocytosis. Toxins that directly target actin molecules rapidly disrupt the entire actin cytoskeleton, which is concomitant with a brutal and drastic rupture of cell barrier integrity. In contrast, toxins and virulence factors acting on Rho-GTPases modulate specific cellular functions, thus allowing pathogens to alter certain intercellular junctions or to cross through cell barriers and escape host mechanisms of defense (phagocytosis in non-killer cells, intra-/inter-cellular motility, and antiphagosytosis directed towards macrophages and polynuclear cells). How have procaryotes evolved such factors that mimic eucaryotic molecules for actin regulation? Structure analysis shows that procaryotes synthesize original molecules that differ from their eucaryotic partners, yet there is a retention of conserved active motifs. Although the basis of this coevolution and their selective pressure remains largely unknown, it is apparent that such molecular mimicry leads to pathogen "success." As time progresses, there is no doubt that additional pathogens and novel methods by which they exploit the eucaryotic actin system will be discovered by various laboratories throughout the world. Now, how can we use all this knowledge to strike back?

## REFERENCES

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003). Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J. Cell Biol.* **160**, 321–328.

Adamson, R.H., Curry, F.E., Adamson, G., Liu, B., Jiang, Y., Aktories, K., Barth, H., Daigeler, A., Golenhofen, N., Ness, W. and Drenckhahn, D. (2002). Rho and rho kinase modulation of barrier properties: cultured endothelial cells and intact microvessels of rats and mice. *J. Physiol*. **539**, 295–308.

Aepfelbacher, M., Trasak, C., Wilharm, G., Wiedmann, A., Trülzsch, K., Krauss, K., Gierschik, P. and Heesemann, J. (2003). Characterization of YopT effects on Rho-GTPases in *Yersinia enterocolitica*-infected cells. *J. Biol. Chem*. **278**, 33217–33223.

Aepfelbacher, M., Zumbihi, R., Ruckdeschel, K., Rouot, B. and Heesemann, J. (2000). Translocated Toxins and Modulins of *Yersinia*. In: *Bacterial Protein Toxins*, eds. K. Aktories and I. Just, Berlin: Springer, 669–689.

Aktories, K. (2000). Bacterial protein toxins as tools in cell biology and pharmacology. In: *Cellular Microbiology* (eds. P. Cossart, P. Boquet, S. Normark and R. Rappuoli) pp. 221–237. ASM Press, Washington, D. C.

Aktories, K. and Koch, G. (1995). Modification of actin and Rho proteins by clostridial ADP-ribosylating toxins. In: *Bacterial Toxins and Virulence Factors in Disease*, vol. 8 (eds. J. Moss, B. Iglewski, M. Vaughan and A.T. Tu), pp. 491–520. Marcel Dekker, New York.

Aktories, K., Weller, U. and Chatwal, G.S. (1987). *Clostridium botulinum* type C produces a novel ADP-ribosyltransferase distinct from botulinum C2 toxin. *FEBS Let*. **212**, 109–113.

Ally, S., Sauer, N.J., Loureiro, J.J., Snapper, S.B., Gertler, F.B. and Goldberg, M.B. (2004). *Shigella* interactions with the actin cytoskeleton in the absence of ENA/VASP family proteins. *Cell. Microbiol*. **6**, 355–366.

Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J. and Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* **300**, 1430–1434.

Andor, A., Trülzsch, K., Essler, M., Roggenkamp, A., Wiedemann, A., Heesemann, J. and Aepfelbacher, M. (2001). YopE of *Yersinia*, a GAP for Rho-GTPases, selectively modulates Rac-dependent actin structures in endothelial cells. *Cell. Microbiol*. **3**, 301–310.

Auerbuch, V., Loureiro, J.J., Gertler, F.B., Theriot, J.A. and Portnoy, D.A. (2003). Ena/VASP proteins contribute to *Listeria monocytogenes* pathogenesis by controlling temporal and spatial persistence of bacterial actin-based motility. *Mol. Microbiol*. **49**, 1361–1375.

Bachmeyer, C., Benz, R., Barth, H., Aktories, K., Gibert, M. and Popoff, M.R. (2001). Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes and Vero cells: inhibition of channel function by chloroquine and related compounds *in vitro* and toxin action *in vivo*. *FASEB J*. **15**, 1658–1660.

Barbier, J., Popoff, M.R. and Molgo, J. (2004). Degeneration and regeneration of murine skeletal neuromuscular junctions after intramuscular injection with a sublethal dose of *Clostridium sordellii* lethal toxin. *Infect. Immun*. **72**, 3120–3128.

Barbieri, J.T. (2000). *Pseudomonas aeruginosa* exoenzyme S, a bifunctional type-III secreted cytotoxin. *Int. J. Med. Microbiol*. **290**, 381–387.

Barbieri, J.T., Riese, M.J. and Aktories, K. (2002). Bacterial toxins that modify the actin cytoskeleton. *Annu. Rev. Cell Dev. Biol*. **18**, 315–344.

Barth, H., Aktories, K., Popoff, M.R. and Stiles, B.G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol. Mol. Biol. Rev*. **68**, 373–402.

Barth, H., Blöcker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R. and Aktories, K. (2000). Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification. *J. Biol. Chem*. **275**, 18704–18711.

Barth, H., Hofmann, F., Olenik, C., Just, I. and Aktories, K. (1998). The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect. Immun*. **66**, 1364–1369.

Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R. and Aktories, K. (2001). Low pH-induced formation of ion channels by *Clostridium difficile* toxin B in target cells. *J. Biol. Chem*. **276**, 10670–10676.

Barth, H., Roebling, R., Fritz, M. and Aktories, K. (2002). The binary *Clostridium botulinum* C2 toxin as a protein delivery system. *J. Biol. Chem*. **277**, 5074–5081.

Barz, C., Abahji, T.N., Trülzsch, K., and Heesemann, J. (2000). The *Yersinia* Ser/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1. *FEBS Lett*. **482**, 139–143.

Bazzoni, G. and Dejana, E. (2004). Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol. Rev*. **84**, 869–901.

Bishop, A.L. and Hall, A. (2000). Rho-GTPases and their effector proteins. *Biochem. J*. **348**, 241–255.

Black, D.S. and Bliska, J.B. (2000). The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. *Mol. Microbiol*. **37**, 515–527.

Blöcker, D., Barth, H., Maier, E., Benz, R., Barbieri, J.T. and Aktories, K. (2000). The C terminus of component C2II of *Clostridium botulinum* C2 toxin is essential for receptor binding. *Infect. Immun*. **68**, 4566–4573.

Bluetow, L., Flatau, G., Chiu, K., Boquet, P. and Ghosh, P. (2001). Structure of the Rho-activating domain of *Escherichia coli* cytotoxic necrotizing factor 1. *Nature Struct. Biol*. **8**, 584–588.

Böhmer, J., Jung, M., Sehr, P., Fritz, G., Popoff, M. R., Just, I. and Aktories, K. (1996). Active site mutation of the C3-like ADP-ribosyltransferase from *Clostridium limosum*-analysis of glutamic acid 174. *Biochemistry* **35**, 282–289.

Boquet, P. (2001). The cytotoxic necrotizing factor 1 (CNF1) from *Escherichia coli*. *Toxicon* **39**, 1673–1680.

Bourmeyster, N., Strasia, M.J., Garin, J., Gagnon, J., Boquet, P. and Vignais, P. (1992). Copurification of Rho protein and the Rho GDP dissociation inhibitor from bovine neutrophil cytosol. Effects of phosphoinositides on Rho ADP-ribosylation by the C3 exoenzyme of *Clostridium botulinum*. *Biochemistry* **31**, 12863–12869.

Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, J.A.T. (2001). Identification of the cellular receptor for anthrax toxin. *Nature (London)* **414**, 225–229.

Braga, V.M., Machesky, L.M., Hall, A. and Hotchin, N.A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol*. **137**, 1421–1431.

Braga, V.M.M., Maschio, A.D., Machesky, L. and Dejna, E. (1999). Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol. Biol. Cell* **10**, 9–22.

Brito, G.A.C., Fujii, J., Carneiro-Filho, B.A., Lima, A.A., Obrig, T. and Guerrant, R.L. (2002). Mechanism of *Clostridium difficile* toxin A-induced apoptosis in T84 cells. *J. Infect. Dis*. **186**, 1438–1447.

Brubaker, R.R. (2003). Interleukin-10 and inhibition of innate immunity to Yersiniae: roles of Yops and LcrV (V antigen). *Infect. Immun*. **71**, 3673–3681.

Buchwald, G., Friebel, A., Galan, J.E., Hardt, W.D., Wittinghofer, A. and Scheffzek, K. (2002). Structural basis for the reversible activation of a Rho protein by the bacterial toxin SopE. *EMBO J*. **21**, 3286–3295.

Burr, S.E., Stuber, K. and Frey, J. (2003). The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida*, subsp. *salmonicida* is translocated via a type-III secretion pathway. *J. Bacteriol*. **185**, 6583–6591.

Burridge, K. and Wennerberg, K. (2004). Rho and Rac take center stage. *Cell* **116**, 167–179.

Campellone, K.G. and Leong, J.M. (2003). Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. *Cur. Opin. Microbiol*. **6**, 82–90.

Campellone, K.G., Robbins, D. and Leong, J.M. (2004). EspFu is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Develop. Cell* **7**, 217–228.

Carbajal, J.M. and Schaeffer, R.C. (1999). RhoA inactivation enhances endothelial barrier function. *Am. J. Physiol*. **277**, C955–C964.

Carlier, M.F. (1998). Control of actin dynamics. *Cur. Biol*. **10**, 45–51.

Carlier, M.F., Le Clainche, C., Wiesner, S. and Pantaloni, D. (2003). Actin-based motility: from molecules to movement. *BioEssays* **25**, 336–345.

Caron, E. and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho-GTPases. *Science* **282**, 1717–1721.

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989). The mammalian G protein *rhoC* is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J*. **8**, 1087–1092.

Chattopadhyay, A., Bhatnagar, N.B. and Bhatnagar, R. (2004). Bacterial insecticidal toxins. *Crit. Rev. Microbiol*. **30**, 33–54.

Chaves-Olarte, E., Florin, I., Boquet, P., Popoff, M.R., von Eichel Streiber, C. and Thelestam, M. (1996). UDP-glucose deficiency in a mutant cell line protects against glucosyltransferase toxins from *Clostridium difficile* and *Clostridium sordellii*. *J. Biol. Chem*. **271**, 6925–6932.

Chen, L.M., Hobble, S. and Galan, J. E. (1996). Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses. *Science* **274**, 2115–2118.

Chen, M.L., Pothoulakis, C. and LaMont, J.T. (2002). Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to *Clostridium difficile* toxin A. *J. Biol. Chem*. **277**, 4247–4254.

Clifton, D.R., Fields, K.A., Grieshaber, S.S., Dooley, C.A., Fisher, E.R., Mead, D.J., Carabeo, R. A. and Hackstadt, T. (2004). A chlamydia type-III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin. *Proc. Natl. Acad. Sci. USA* **101**, 10166–10171.

Cooper, J.A. and Schafer, D.A. (2000). Control of actin assembly and disassembly at filament ends. *Cur. Op. Cell Biol*. **12**, 97–103.

Cornelis, G.R. (2002). The *Yersinia* Ysc-Yop type-III weaponry. *Nature Rev*. **1**, 742–752.

Cossart, P. and Sansonetti, P.J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304**, 242–248.

Cudmore, S., Reckmann, I. and Way, M. (1997). Viral manipulations of the actin cytoskeleton. *Trends Microbiol*. **5**, 142–148.

Cunningham, K., Lacy, D.B., Mogridge, J. and Collier, R.J. (2002). Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc. Natl. Acad. Sci USA* **99**, 7049–7053.

Czech, A., Yamaguchi, T., Bader, L., Linder, S., Kamisnki, K., Sugai, M. and Aepfelbacher, M. (2001). Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical *Staphylococcus aureus* isolates. *J. Infec. Dis*. **184**, 785–788.

Dos Remedios, C.G., Kekic, C.M., Tsubakhara, M., Berry, D.A. and Nosworthy, N. J. (2002). Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol. Rev*. **83**, 433–473.

Doye, A., Mettouchi, A., Bossis, G., Clément, R., Buisson-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P. and Lemichez, E. (2002). CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho-GTPase activation for bacterial host cell invasion. *Cell* **111**, 553–564.

Dumenil, G., Sansonetti, P. and Tran Van Nhieu, G. (2000). Src tyrosine kinase activity down-regulates rho-dependent responses during *Shigella* entry into epithelial cells and stress fiber formation. *J. Cell Sci*. **113**, 71–80.

Eckhardt, M., Barth, H., Blöcker, D. and Aktories, K. (2000). Binding of *Clostridium botulinum* C2 toxin to asparagine-linked complex and hybrid carbohydrates. *J. Biol. Chem*. **275**, 2328–2334.

Egile, C., Loisel, T., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. and Carlier, M. (1999). Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J. Cell Biol*. **146**, 1319–1332.

Ermert, L., Duncker, H.R., Brückner, H., Grimminger, F., Hansen, T., Rössig, R., Aktories, K. and Seeger, W. (1997). Ultrastructural changes of lung capillary endothelium in response to botulinum C2 toxin. *J. Appl. Physiol*. **82**, 382–388.

Essler, M., Hermann, K., Amano, M., Kaibuchi, K., Heesemann, J., Weber, P.C. and Aepfelbacher, M. (1998). *Pasteurella multocida* toxin increases endothelial permeability via Rho kinase and myosin light chain phosphatase. *J. Immunol*. **161**, 5640–5646.

Evans, H.R., Sutton, J.M., Holloway, D.E., Ayriss, J., Shone, C. C. and Acharya, K.R. (2003). The crystal structure of C3stau2 from *Staphylococcus aureus* and its complex with NAD. *J. Biol. Chem*. **278**, 45924–45930.

Evdokimov, A., Tropea, J.E., Routzahn, K.M. and Waugh, D.S. (2002). Crystal structure of the *Yersinia pestis* GTPase activator YopE. *Prot. Sci*. **11**, 401–408.

Fields, K.A., Mead, D.J., Dooley, C.A. and Hackstadt, T. (2003). *Chlamydia trachomatis* type-III secretion: evidence for a functional apparatus during early-cycle development. *Mol. Microbiol*. **48**, 671–683.

Fiorentini, C., Fabbri, A., Flatau, G., Donelli, G., Mataresse, P., Lemichez, E., Falzano, L. and Boquet, P. (1997). *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1), a toxin that activates the Rho-GTPase. *J. Biol. Chem*. **272**, 19532–19537.

Friebel, A., Ilchmann, H., Aepfelbancher, M., Ehrbar, K., Machleidt, W. and Hardt, W.D. (2001). SopE and SopE2 from *Salmonella typhimurium* activate different sets of Rho-GTPases of the host cell. *J. Biol. Chem*. **276**, 34035–34040.

Frischknecht, F., Moreau, V., Röttger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G. and Way, M. (1999). Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signaling. *Nature* **401**, 926–929.

Fu, H., Coburn, J. and Collier, R.J. (1993). The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. *Proc. Natl. Acad. Sci. USA* **90**, 2320–2324.

Fu, Y. and Galan, J.E. (1999). A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature* **401**, 293–297.

Fujihara, H., Walker, L.A., Gong, M.C., Lemichez, E., Boquet, P., Somlyo, A.V. and Somlyo, A.P. (1997). Inhibition of RhoA translocation and calcium sensitization by *in vivo* ADP-ribosylation with the chimeric toxin DC3B. *Mol. Biol. Cell* **8**, 2437–2447.

Fukata, M. and Kaibuchi, K. (2001). Rho-family GTPases in cadherin-mediated cell-cell adhesion. *Nature Rev. Mol. Cell Biol*. **2**, 887–897.

Fullner, K.J., Lencer, W.I. and Mekalanos, J.J. (2000). *Vibrio cholerae*-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. *Infect. Immun*. **69**, 6310–6317.

Fullner, K.J. and Mekalanos, J.J. (2000). *In vivo* covalent cross-linking of cellular actin by the *Vibrio cholerae* RTX toxin. *EMBO J*. **19**, 5315–5323.

Galan, J.E. (2001). *Salmonella* interactions with host cells: type-III secretion at work. *Ann. Rev. Cell Dev. Biol*. **17**, 53–86.

Galan, J.E. and Zhou, D. (2000). Striking a balance: modulation of the actin cytoskeleton by *Salmonella*. *Proc. Natl. Acad. Sci. USA* **97**, 8754–8761.

Galkin, V.E., Orlova, A., VanLoock, M.S., Zhou, D., Galan, J.E. and Egelman, E. H. (2002). The bacterial protein SipA polymerizes G-actin and mimics muscle nebulin. *Nature Struct. Biol*. **9**, 518–521.

Ganessan, A.K., Mende-Mueller, L., Selzer, J. and Barbieri, J. T. (1999). *Pseudomonas aeruginosa* exoenzyme S, a double ADP-ribosyltransferase, resembles vertebrate mono-ADP-ribosyltransferases. *J. Biol. Chem*. **274**, 9503–9508.

Garrity-Ryan, L., Shafikhani, S., Balachandran, P., Nguyen, L., Oza, J., Jakobsen, T., Sargent, J., Fang, X., Cordwell, S., Matthay, M. A. and Engel, J.N. (2004). The ADP ribosyltransferase domain of *Pseudomonas aeruginosa* ExoT contributes to its biological activities. *Infect. Immun*. **72**, 546–558.

Genth, H., Aktories, K. and Just, I. (1999). Monoglucosylation of RhoA at threonine 37 blocks cytosol membrane recycling. *J. Biol. Chem*. **274**, 29050–29056.

Gibert, M., Petit, L., Raffestin, S., Okabe, A. and Popoff, M. R. (2000). *Clostridium perfringens* iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. *Infect. Immun*. **68**, 3848–3853.

Goehring, U.M., Schmidt, G., Pederson, K.J., Aktories, K. and Barbieri, J.T. (1999). The N-terminal domain of *Pseudomonas aeruginosa* exoenzymes is a GTPase-activating protein for Rho-GTPases. *J. Biol. Chem*. **274**, 36369–36372.

Gotoh, H., Okada, N., Kim, Y.G., Shiraishi, K., Hirami, N., Haneda, T., Kurita, A., Kikuchi, A. and Danbara, H. (2003). Extracellular secretion of the virulence plasmid-encoded ADP-ribosyltransferase SpvB in *Salmonella*. *Microb. Pathog*. **34**, 227–238.

Gouin, E., Eglle, C., Dehoux, P., Villiers, V., Adams, J., Gertler, F., Li, R. and Cossart, P. (2004). The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex. *Nature* **427**, 457–461.

Habermann, B., Mohr, C., Just, I. and Aktories, K. (1991). ADP-ribosylation and de-ADP-ribosylation of the Rho protein by *Clostridium botulinum* exoenzyme C3. Regulation by EDTA, guanine nucleotides, and pH. *Biochim. Biophys. Acta* **1077**, 253–258.

Han, S., Arvai, A.S., Clancy, S.B. and Tainer, J.A. (2001). Crystal structure and novel recognition motif of Rho ADP-ribosylating C3 exoenzyme from *Clostridium botulinum*: structural insights for recognition specificity and catalysis. *J. Mol. Biol*. **305**, 95–107.

Han, S., Craig, J.A., Putnam, C.D., Carozzi, N.B. and Tainer, J.A. (1999). Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nature Struct. Biol*. **6**, 932–936.

Hardt, W.D., Chen, L.M., Schuebel, K.E., Bustelo, X.R. and Galan, J.E. (1998). *Salmonella typhimurium* encodes an activator of Rho-GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93**, 815–826.

Harhaj, N.S. and Antonetti, D.A. (2004). Regulation of tight junctions and loss of barrier function in pathophysiology. *Int. J. Biochem. Cell Biol*. **36**, 1206–1237.

Hayward, R.D. and Koronakis, V. (1999). Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella*. *EMBO J*. **18**, 4926–4934.

Hayward, R.D. and Koronakis, V. (2002). Direct modulation of the host cell cytoskeleton by *Salmonella* actin-binding proteins. *Trends Cell Biol*. **12**, 15–20.

He, D., Hagen, S.J., Pothoulakis, C., Chen, M., Medina, N.D., Warny, M. and Lamont, J.T. (2000). *Clostridium difficile* toxin A causes early damage to mitochondria in cultured cells. *Gastroenterology* **119**, 139–150.

Hecht, G., Koutsouris, A., Pothoulakis, C., Lamont, J.T. and Madara, J.L. (1992). *Clostridium difficile* toxin B disrupts the barrier function of T84 monolayers. *Gastroenterology* **102**, 416–423.

Hecht, G., Pothoulakis, C., Lamont, J.T. and Madara, J.L. (1988). *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J. Clin. Invest*. **82**, 1516–1524.

Herreros, J., Ng, T. and Schiavo, G. (2001). Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. *Mol. Biol. Cell* **12**, 2947–2960.

Higashide, W., Dai, S., Hombs, V.P. and Zhou, D. (2002). Involvement of SipA in modulating actin dynamics during *Salmonella* invasion into cultured epithelial cells. *Cell. Microbiol*. **4**, 357–365.

Higgs, H.N. and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem*. **70**, 649–676.

Hirase, T., Kawashima, S., Wong, E.Y., Ueyama, T., Rikitake, Y., Tsukita, S., Yokoyama, M. and Staddon, J.M. (2001). Regulation of tight junction permeability and occludin phosphorylation by RhoA-p160ROCK-dependent and -independent mechanisms. *J. Biol. Chem*. **276**, 10423–10431.

Hopkins, A.M., Walsh, S.V., Verkade, P., Boquet, P. and Nusrat, A. (2002). Constitutive activation of Rho proteins by CNF1 influences tight junction structure and epithelial barrier function. *J. Cell Sci*. **116**, 725–742.

Ishida, Y., Maegawa, T., Kondo, T., Kimura, A., Iwakura, Y., Nakamura, S. and Mukaida, N. (2004). Essential involvement of IFN-gamma in *Clostridium difficile* toxin A-induced enteritis. *J. Immunol*. **172**, 3018–3025.

Jennison, A. V. and Verma, N. K. (2004). *Shigella flexneri* infection: pathogenesis and vaccine development. *FEMS Microbiol. Rev*. **28**, 43–58.

Jepson, M.A., Pellegrin, S., Peeto, L., Banbury, D.N., Leard, A.D., Mellor, H. and Kenny, B. (2003). Synergistic roles for the Map and Tir effector molecules in mediating uptake of enteropathogenic *Escherichia coli* (EPEC) into non-phagocytic cells. *Cell. Microbiol*. **5**, 773–783.

Johal, S.S., Solomon, K., Dodson, S., Borriello, S.P. and Mahida, Y.R. (2004). Differential effects of varying concentrations of *Clostridium difficile* toxin A on epithelial barrier function and expression of cytokines. *J. Infect. Dis*. **189**, 2110–2119.

Jou, T., S., Schneeberger, E.E. and Nelson, W.J. (1998). Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J. Cell Biol*. **142**, 101–115.

Just, I., Hofmann, F. and Aktories, K. (2000). Molecular mechanism of action of the large clostridial cytotoxins. In: *Bacterial Protein Toxins* (eds. K. Aktories and I. Just), pp. 307–331. Springer, Berlin

Just, I., Selzer, J., Jung, M., Van Damme, J., Vandekerckhove, J. and Aktories, K. (1995). Rho-ADP-ribosylating exoenzyme from *Bacillus cereus*. Purification, characterization, and identification of the NAD-binding site. *Biochemistry* **34**, 334–340.

Kalman, D., Weiner, O.D., Goosney, D.L., Sedat, J.W., Finlay, B.B., Abo, A. and Bishop, J. M. (1999). Enteropathogenic *E. coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nature Cell Biol*. **1**, 389–391.

Kenny, B., Ellis, S., Leard, A., Warawa, J., Mellor, H. and Jepson, M. (2002). Coordinate regulation of distinct host cell signaling pathways by multifunctional enteropathogenic *Escherichia coli* effector molecules. *Mol. Microbiol*. **44**, 1095–1107.

Kiefer, G., Lerner, M., Sehr, P., Just, I. and Aktories, K. (1996). Cytotoxic effects by microinjection of ADP-ribosylated skeletal muscle G-actin in PtK2 cells in the absence of *Clostridium perfringens* iota toxin. *Med. Microbiol. Immunol*. **184**, 175–180.

Knapp, O., Benz, R., Gibert, M., Marvaud, J.C. and Popoff, M.R. (2002). Interaction of *Clostridium perfringens* iota-toxin with lipid bilayer membranes. *J. Biol. Chem*. **277**, 6143–6152.

Koch, G., Benz, C., Schmidt, G., Olenik, C. and Aktories, K. (1997). Role of Rho protein in lovastatin-induced breakdown of actin cytoskeleton. *J. Pharmacol. Exper. Ther*. **283**, 901–909.

Kotani, H., Takaishi, K., Sasaki, T. and Takai, Y. (1997). Rho regulates association of both the ERM family and vinculin with the plasma membrane in MDCK cells. *Oncogene* **14**, 1705–1713.

Krall, R., Schmidt, G., Aktories, K. and Barbieri, J.T. (2000). *Pseudomonas aeruginosa* exoT is a Rho-GTPase-activating protein. *Infect. Immun*. **68**, 6066–6068.

Kurita, A., Gotoh, H., Eguchi, M., Okada, N., Matsuura, S., Matsui, H., Danbara, H. and Kikuchi, Y. (2003). Intracellular expression of the *Salmonella* plasmid virulence protein SpvB causes apoptotic cell death in eukaryotic cells. *Microb. Pathog*. **35**, 43–48.

Law, M., Hollinshead, M., Lee, H.J. and Smith, G.L. (2004). Yaba-like disease virus protein Y144R, a member of the complement control protein family, is present on enveloped virions that are associated with virus-induced actin tails. *J. Gen. Virol*. **85**, 1279–1290.

Lee, H.J. and Galan, J.E. (2004). *Salmonella* type-III secretion-associated chaperons confer secretion-pathway specificity. *Mol. Microbiol*. **51**, 483–495.

Lesnick, M.L., Reiner, N.E., Fierer, J. and Guiney, D.G. (2001). The *Salmonella spvB* virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Mol. Microbiol*. **39**, 1464–1470.

Lilic, M., Galkin, V.E., Orlova, A., VanLoock, M.S., Egelman, E.H. and Stebbins, C. E. (2003). *Salmonella* SipA polymerizes actin by stapling filaments with non-globular protein arms. *Science* **301**, 1918–1921.

Lin, S.L., Le, T.X. and Cowen, D.S. (2003). SptP, a *Salmonella typhimurium* type-III-secreted protein, inhibits the mirogen-activated protein kinase pathway by inhibiting Raf activation. *Cell. Microbiol*. **5**, 267–275.

Lockman, H.A., Gillespie, R.A., Baker, B.D. and Shakhnovich, E. (2002). *Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor. *Infect. Immun*. **70**, 2708–2714.

Loisel, T., Boujeemaa, R., Pantaloni, D. and Carlier, M. (1999). Reconstitution of actin-based motility of *Listeria* and *Shigella* using pure proteins. *Nature* **401**, 613–616.

Löwe, J., van der Ent, F. and Amos, L. A. (2004). Molecules of the bacterial cytoskeleton. *Annu. Rev. Biophys. Biomol. Struct*. **33**, 177–198.

Marvaud, J.C., Smith, T., Hale, M.L., Popoff, M.R., Smith, L.A. and Stiles, B.G. (2001). *Clostridium perfringens* iota-toxin: mapping of receptor binding and Ia docking domains on Ib. *Infect. Immun*. **69**, 2435–2441.

Marvaud, J.C., Stiles, B.G., Chenal, A., Gillet, D., Gibert, M., Smith, L. A. and Popoff, M.R. (2002). *Clostridium perfringens* iota toxin: mapping of the Ia domain involved in docking with Ib and cellular internalization. *J. Biol. Chem*. **277**, 43659–43666.

Masignani, V., Balduci, E., Serruto, D., Veggi, D., Arico, B., Comanducci, M., Pizza, M. and Rappuoli, R. (2004). In silico identification of novel bacterial ADP-ribosyltransferases. *Int. J. Med. Microbiol*. **293**, 471–478.

Matsui, H., Bacot, C.M., Garlington, W.A., Doyle, T.J., Roberts, S. and Gulig, P.A. (2001). Virulence plasmid-borne *spvB* and *spvC* genes can replace the 90-kilobase plasmid in conferring virulence to *Salmonella enterica* serovar Typhimurium in subcutaneously inoculated mice. *J. Bacteriol*. **183**, 4352–4658.

McClane, B.A. (2001). The complex interactions between *Clostridium perfringens* enterotoxin and epithelial tight junctions. *Toxicon* **39**, 1781–1791.

McClane, B.A. and Rood, J.I. (2001). Clostridial toxins involved in human enteric and histotoxic infections. In: *Clostridia* (eds. H. Bahl and P. Dürre), pp. 169–209. Willey-VCH, Weinheim.

McNamara, B.P., Koutsouris, A., O'Connell, C.B., Nougayréde, J.P., Donnenberg, M.S. and Hecht, G. (2001). Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *J. Clin. Invest*. **107**, 621–629.

Ménétrey, J., Flatau, G., Stura, E.A., Charbonnier, J.B., Gas, F., Teulon, J.M., Le Du, M.H., Boquet, P. and Ménez, A. (2002). NAD binding induces conformational changes in Rho ADP-ribosylating *Clostridium botulinum* C3 exoenzyme. *J. Biol. Chem*. **277**, 30950–30957.

Mogridge, J., Cuningham, K., Lacy, D.B., Mourez, M. and Collier, R.J. (2002a). The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. *Proc. Natl. Acad. Sci. USA* **99**, 7045–7048.

Mogridge, J., Cunningham, K. and Collier, R.J. (2002b). Stoichiometry of anthrax toxin complexes. *Biochemistry* **41**, 1079–1082.

Monnazzi, L.G., Carlos, I.Z. and de Madeiros, B.M. (2004). Influence of *Yersinia pseudotuberculosis* outer proteins (Yops) on interleukin-12, tumor necrosis factor alpha and nitric oxide production by peritoneal macrophages. *Immunol. Lett*. **94**, 91–98.

Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D. and Way, M. (2000). A complex of N-WASP and WIP integrates signaling cascades that lead to actin polymerization. *Nature Cell Biol*. **2**, 441–448.

Mukherjee, K., Parashuraman, S., Raje, M. and Mukhopadhyay, A. (2001). SopE acts as an Rab5-specific nucleotide exchange factor and recruits non-prenylated Rab5 on *Salmonella*-containing phagosomes to promote fusion with early endosomes. *J. Biol. Chem*. **276**, 23607–23615.

Murli, S., Watson, R.O. and Galan, J.E. (2001). Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of *Salmonella* with host cells. *Cell. Microbiol*. **3**, 795–810.

Nassif, X., Bourdoulous, S., Eugène, E. and Couraud, P.O. (2002). How do extracellular pathogens cross the blood-brain barrier? *Trends Microbiol*. **10**, 227–232.

Naumann, M. and Crabtree, J.E. (2004). *Helicobacter pylori*-induced epithelial cell signaling in gastric carcinogenesis. *Trends Microbiol*. **12**, 29–36.

Nejedik, L., Pierfelice, T. and Geisser, J.R. (2004). Actin distribution is disrupted upon expression of *Yersinia* YopO/YpkA in yeast. *Yeast* **21**, 759–768.

Niebuhr, K., Giuriato, S., Pedron, T., Philpott, D.J., Gaits, F., Sable, J., Sheetz, M.P., Parsot, C., Sansonetti, P.J. and Payrastre, B. (2002). Conversion of PtdIns(4,5)P2 into PtdIns(5)P by the *S. flexneri* effector IpgD reorganizes host cell morphology. *EMBO J*. **21**, 5069–5078.

Nusrat, A., Giry, M., Turner, J.R., Colgan, S.P., Parkos, C.A., Carnes, D., Lemichez, E., Boquet, P. and Madara, J.L. (1995). Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. *Proc. Natl. Acad. Sci. USA* **92**, 10629–10633.

Ohishi, I. (2000). Structure and function of actin-adenosine-diphosphate-ribosylating toxins. In: *Bacterial Protein Toxins*, vol. 145 (eds. K. Aktories and I. Just), pp. 253–273 Springer, Berlin.

Ohishi, I. and Odagiri, Y. (1984). Histopathological effects of botulinum C2 toxin on mouse intestines. *Infect. Immun*. **43**, 54–58.

Ottlinger, M.E. and Lin, S. (1988). *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultured cells. *Exp. Cell Res*. **174**, 215–229.

Paavilainen, V.O., Bertling, E., Falck, S. and Lappalainen, P. (2004). Regulation of cytoskeletal dynamics by actin-monomer-binding proteins. *Trends Cell Biol*. **14**, 386–394.

Pederson, K.J., Vallis, A., Aktories, K., Frank, D.W. and Barbieri, J.T. (2004). The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small molecular-weight GTP-binding proteins. *Mol. Microbiol*. **32**, 393–401.

Perrez-Moreno, M., Jamora, C. and Fuchs, E. (2003). Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112**, 535–548.

Petit, L., Maier, E., Gibert, M., Popoff, M.R. and Benz, R. (2001). *Clostridium perfringens* epsilon-toxin induces a rapid change in cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J. Biol. Chem* **276**, 15736–15740.

Petosa, C., Collier, J.R., Klimpel, K.R., Leppla, S.H. and Liddington, R. C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature (London)* **385**, 833–838.

Pfeifer, G., Schirmer, J., Leemhuuis, J., Busch, C., Meyer, D.K., Aktories, K. and Barth, H. (2003). Cellular uptake of *Clostridium difficile* toxin B: translocation of the N-terminal catalytic domain into the cytosol of eukaryotic cells. *J. Biol. Chem*. **278**, 44535–44541.

Pizza, M., Masignani, V. and Rappuoli, R. (1999). Molecular, functional, and evolutionary aspects of ADP-ribosylating toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), pp. 45–72 Academic Press, London.

Plant, L. and Jonsson, A. B. (2003). Contacting the host: insights and implications of pathogenic *Neisseria* cell interactions. *Scand. J. Infect. Dis*. **35**, 608–613.

Pollard, T.D., Blanchoin, L. and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu. Rev. Biophys. Biomol. Struct*. **29**, 545–576.

Pollard, T.D. and Borisy, G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112,** 453–465.

Popoff, M.R. (2000). Molecular biology of actin-ADP-ribosylating toxins. In: *Bacterial Protein Toxins* (eds. K. Aktories and I. Just), pp. 275–302. Springer, Berlin.

Pothoulakis, C. and Lamont, J.T. (2001). Microbes and microbial toxins: paradigms for microbial-mucosa interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. *Am. J. Physiol. Gastrointest. Liver Physiol*. **280,** G178–G183.

Qa'dan, M., Spyres, L.M. and Ballard, J.D. (2000). pH-induced conformational changes in *Clostridium difficile* toxin B. *Infect. Immun*. **68,** 2470–2474.

Qa'dan, M., Spyres, L.M. and Ballard, J.D. (2001). pH-induced cytopathic effects of *Clostridium sordellii* lethal toxin. *Infect. Immun*. **69,** 5487–5493.

Ren, X.D., Bokoch, G.M., Traynor-Kaplan, A., Jenkins, G.H., Anderson, R.A. and Schwartz, M.A. (1996). Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in swiss **3**T3 cells. *Mol. Biol. Cell* **7,** 435–442.

Reuner, K.H., Dunker, P., van der Does, A., Wiederhold, M., Just, I., Aktories, K. and Katz, N. (1996). Regulation of actin synthesis in rat hepatocytes by cytoskeleton rearrangements. *Eur. J. Cell Biol*. **69,** 189–196.

Richard, J.F., Mainguy, G., Gibert, M., Marvaud, J.C., Stiles, B.G. and Popoff, M.R. (2002). Transcytosis of iota toxin across polarized CaCo-2 cell monolayers. *Mol. Microbiol*. **43,** 907–917.

Richard, J.F., Petit, L., Gibert, M., Marvaud, J.C., Bouchaud, C. and Popoff, M.R. (1999). Bacterial toxins modifying the actin cytoskeleton. *Internatl. Microbiol*. **2,** 185–194.

Rubin, E.J., Gill, D.M., Boquet, P. and Popoff, M.R. (1988). Functional modification of a 21-kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. *Mol. Cell. Biol*. **8,** 418–426.

Sah, V.P., Seasholtz, T.M., Sagi, S.A. and Brown, J.H. (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annu. Rev. Pharmacol. Toxicol*. **40,** 459–489.

Samarin, S., Romero, S., Kocks, C., Didry, D., Pantaloni, D. and Carlier, M. (2003). How VASP enhances actin-based motility. *J. Cell Biol*. **163,** 131–142.

Sanfilippo, L., Li, C.K., Seth, R., Balwin, T.J., Menozzi, M.G. and Mahida, Y.R. (2000). *Bacteroides fragilis* enterotoxin induces the expression of IL-8 and transforming growth factor-beta (TGF-β) by human colonic epithelial cells. *Clin. Exp. Immunol*. **119,** 456–463.

Sansonetti, P. (2002). Host-pathogen interactions: the seduction of molecular cross talk. *Gut* **50,** ii2–ii8.

Savidge, T.C., Pan, W.H., Newman, P., O'Brien, M., Anton, P.M. and Pothoulakis, C. (2003). *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. *Gastroenterology* **125,** 413–420.

Schirmer, J. and Aktories, K. (2004). Large clostridial toxins: cellular biology of Rho/Ras-glucosylating toxins. *Biochem. Biophys. Acta* **1673,** 66–74.

Schmid, A., Benz, R., Just, I. and Aktories, K. (1994). Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes. *J. Biol. Chem*. **269,** 16706–16711.

Schmidt, G., Goehring, U.M., Schirmer, J., Lerm, M. and Aktories, K. (1999). Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for Rho-GTPases. *J. Biol. Chem*. **274,** 31875–31881.

Schneeberger, E.E. and Lynch, R.D. (2004). The tight junction: a multifunctional complex. *Am. J. Physiol. Cell Physiol*. **286,** C1213–C1228.

Sears, C.L. (2001). The toxins of *Bacteroides fragilis*. *Toxicon* **39,** 1737–1746.

Sechi, A.S. and Wehland, J. (2004). ENA/VASP proteins: multifunctional regulators of actin cytoskeleton dynamics. *Front. Biosci*. **9,** 1294–1310.

Sehr, P., Gili, J., Genth, H., Just, I., Pick, E. and Aktories, K. (1998). Glucosylation and ADP ribosylation of Rho proteins: effects on nucleotide binding, GTPase activity, and effector coupling. *Biochemistry* **37,** 5296–5304.

Selbach, M., Moese, S., Backert, S., Jungblut, P.R. and Meyer, T.F. (2004). The *Helicobacter pylori* protein induces tyrosine dephosphorylation of ezrin. *Proteomics* **4,** 2961–2968.

Shao, F., Merritt, P.M., Bao, Z., Innes, R.W. and Dixon, J.E. (2002). A *Yersinia* effector and a *Pseudomonas* avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. *Cell* **109,** 575–588.

Simonovic, I., Rosenberg, J., Koutsouris, A. and Hecht, G. (2000). Enteropathogenic *Escherichia coli* dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. *Cell. Microbiol*. **2,** 305–315.

Singh, U., Mitic, L.L., Wieckowski, E.U., Anderson, J.M. and McClane, B.A. (2001). Comparative biochemical and immunocytochemical studies reveal differences in the effects of *Clostridium perfringens* enterotoxin on polarized CaCo-2 cells *versus* Vero cells. *J. Biol. Chem*. **276,** 33402–33412.

Singh, U., Van Itallie, C.M., Mitic, L.L., Anderson, J.M. and McClane, B.A. (2000). CaCo-2 cells treated with *Clostridium perfringens* enterotoxin form multiple complex species, one of which contains the tight junction protein occludin. *J. Biol. Chem*. **275,** 18407–18417.

Smith, C.L., Khandelwal, P., Keliikuli, K., Zuiderweg, E.R. and Saper, M.A. (2001). Structure of the type-III secretion and substrate-binding domain of *Yersinia* YopH phosphatase. *Mol. Microbiol*. **42,** 967–979.

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev*. **9,** 216–234.

Southwick, F.S. and Purich, D.L. (2000). Actin filaments: self-assembly and regulatory interactions. In: *Cellular Microbiology* (eds. P. Cossart, P. Boquet, S. Normark and R. Rappuoli), pp. 153–170. ASM Press, Washington, D.C.

Stebbins, C.E. and Galan, J.E. (2000). Modulation of host signaling by a bacterial mimic: structure of the *Salmonella* effector SptP bound to Rac1. *Mol. Cell* **6,** 1449–1460.

Stebbins, C.E. and Galan, J.E. (2001). Structural mimicry in bacterial virulence. *Nature* **412,** 701–705.

Stiles, B., Hale, M.L., Marvaud, J.C. and Popoff, M.R. (2000). *Clostridium perfringens* iota toxin: binding studies and characterization of cell surface receptor by fluorescence-activated cytometry. *Infect. Immun*. **68,** 3475–3484.

Stiles, B.G., Hale, M.L., Marvaud, J.C. and Popoff, M.R. (2002). *Clostridium perfringens* iota toxin: characterization of the cell-associated iota b complex. *Biochem. J*. **367,** 801–808.

Stuart, E.S., Webley, W.C. and Norkin, L.C. (2003). Lipid rafts, caveolae, caveolin-1, and entry by *Chlamydiae* into host cells. *Exp. Cell Res*. **287,** 67–78.

Subtil, A., Wyplosz, B., Balana, M.E. and Dautry-Varsat, A. (2004). Analysis of *Chlamydia caviae* entry sites and involvement of Cdc42 and Rac activity. *J. Cell Sci*. **117,** 3923–3933.

Suetsugu, S., Miki, H. and Takenawa, T. (2002). Spatial and temporal regulation of actin polymerization for cytoskeleton formation through Arp2/3 complex and WASP/WAVE proteins. *Cell Mot. Cytosk*. **51,** 113–122.

Sun, J. and Barbieri, J.T. (2004). ExoS Rho-GTPase-activating protein activity stimulates reorganization of the actin cytoskeleton through Rho-GTPase guanine nucleotide dissociation inhibitor. *J. Biol. Chem*. **279,** 42936–42944.

Suzuki, T., Mimuro, H., Miki, H., Takenawa, T., Sasaki, T., Nakanishi, H., Takai, Y. and Sasakawa, C. (2000). Rho family GTPase Cdc42 is essential for actin-based motility of *Shigella* in mammalian cells. *J. Exp. Med*. **191,** 1905–1920.

Takai, Y., Sasaki, T. and Matozaki, T. (2001). Small GTP-binding proteins. *Physiol. Rev*. **81,** 153–208.

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. and Takai, Y. (1997). Regulation of cell-cell adhesion by Rac and Rho small G proteins in MDCK cells. *J. Cell Biol*. **139,** 1047–1059.

Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci*. **114,** 1801–1809.

Tran Van Nhieu, G., Bourdet-Sicard, R., Dumenil, G., Blocker, A. and Sansonetti, P.J. (2000). Bacterial signals and cell responses during *Shigella* entry into epithelial cells. *Cell. Microbiol*. **2,** 187–193.

Tran Van Nhieu, G., Caron, E., Hall, A. and Sansonetti, P.J. (1999). IpaC induces actin polymerization and filopodia formation during *Shigella* entry into epithelial cells. *EMBO J*. **18,** 3249–3262.

Tsuge, H., Nagahama, M., Nishimura, H., Hisatsune, J., Sakaguchi, Y., Itogawa, Y., Katunuma, N. and Sakurai, J. (2003). Crystal structure and site-directed mutagenesis of enzymatic components from *Clostridium perfringens* iota-toxin. *J. Mol. Biol*. **325,** 471–483.

Turner, J.R., Rill, B.K., Carlson, S.L., Carnes, D., Kerner, R., Mrsny, R.J. and Madara, J.L. (1997). Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am. J. Physiol*. **273,** C1378–C1385.

Van Aelst, L. and Souza-Schorey, C.D. (1997). Rho-GTPases and signaling networks. *Genes Develop*. **11,** 2295–2322.

Vandekerckhove, J., Schering, B., Bärmann, M. and Aktories, K. (1987). *Clostridium perfringens* iota toxin ADP-ribosylates skeletal muscle actin in Arg-177. *FEBS Lett*. **255,** 48–52.

Vetter, I. and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. *Science* **294,** 1299–1304.

Vetter, I.R., Hofmann, F., Wohlgemuth, S., Hermann, C. and Just, I. (2000). Structural consequences of monoglucosylation of Ha-Ras by *Clostridium sordellii* lethal toxin. *J. Mol. Biol*. **301,** 1091–1095.

von Pawel-Rammingen, U., Telepnev, M.V., Schmidt, G., Aktories, K., Wolf-Watz, H. and Rosqvist, R. (2000). GAP activity of the *Yersinia* YopE cytotoxin specifically targets the Rho pathway: a mechanism for disruption of actin microfilament structure. *Mol. Microbiol*. **36,** 737–748.

Vouret-Craviari, V., Grall, D., Flatau, G., Pouyssegur, J., Boquet, P. and Van Obberghen-Schilling, E. (1999). Effects of cytotoxic necrotizing factor 1 and lethal toxin on actin cytoskeleton and VE-cadherin localization in human endothelial monolayers. *Infect. Immun*. **67,** 3002–3008.

Vouret-Craviari, V., Boquet, P., Pouysségur, J. and Van Obberghen-Schilling, E. (1998). Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. *Mol. Biol. Cell* **9,** 2639–2653.

Walsh, S.V., Hopkins, A.M., Chen, J., Narumiya, S., Parkos, C.A. and Nusrat, A. (2001). Rho kinase regulates tight junction and is necessary for tight junction assembly in polarized intestinal epithelia. *Gastroenterology*. **121,** 566–579.

Wang, W., Azzau, S., Goldblum, S.E. and Fasano, A. (2000). Human zonulin, a potential modulator of intestinal tight junctions. *J. Cell Sci*. **113,** 4435–4440.

Waschke, J., Baumgartner, W., Adamson, R.H., Zeng, M., Aktories, K., Barth, H., Wilde, C., Curry, F.E. and Drenckhahn, D. (2004). Requirement of Rac activity for maintenance of capillary endothelial barrier properties. *Am. J. Physiol. Heart. Circ. Physiol*. **286,** H394–H401.

Wei, Y., Zhang, Y., Derewenda, U., Liu, X., Minor, W., Nakamoto, R.K., Somlyo, A.V., Somlyo, A.P. and Derewenda, Z.S. (1997). Crystal structure of RhoA-GDP and its functional implications. *Nature Struct. Biol*. **4,** 699–703.

Welch, M., Rosenblatt, J., Skoble, J., Portnoy, D. and Mitchison, T. (1998). Interaction of human Arp2/3 complex and the *Listeria monocytogenes* ActA protein in actin nucleation. *Science* **281,** 105–108.

Wilde, C., Chhatwal, G.S., Schmalzing, G., Aktories, K. and Just, I. (2001). A novel C3-like ADP-ribosyltransferase from *Staphylococus aureus* modifyng RhoE and Rnd3. *J. Biol. Chem*. **276,** 9537–9542.

Wojciak-Stothard, B., Potempa, S., Eichholtz, T. and Ridley, A.J. (2001). Rho and Rac but not Cdc42 regulate endothelial cell permeability. *J. Cell Sci*. **114,** 1343–1355.

Wojciak-Stothard, B. and Ridley, A.J. (2003). Rho-GTPases and the regulation of endothelial permeability. *Vasc. Pharmacol*. **39,** 187–199.

Wu, Z., Nybom, P. and Magnusson, K.E. (2000). Distinct effects of *Vibrio cholerae* haemagglutinin/protease on the structure and localization of the tight junction-associated proteins occludin and ZO-1. *Cell. Microbiol*. **2,** 11–17.

Würtele, M., Wolf, E., Pederson, K.J., Buchwald, G., Ahmadian, M.R., Barbieri, J.T. and Wittinghofer, A. (2001). How the *Pseudomonas aeruginosa* ExoS toxin down-regulates *Rac. Nature Struct. Biol*. **8,** 23–26.

Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B. and Galan, J.E. (2001). A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol*. **39,** 248–259.

Zhou, D., Mooseker, M.S. and Galan, J.E. (1999). Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. *Science* **283,** 2092–2094.

Zigmond, S.H. (2004). Formin-induced nucleation of actin filaments. *Cur. Opin. Cell Biol*. **16,** 99–105.

Zywietz, A., Gohla, A., Schmelz, M., Schultz, G. and Offermanns, S. (2001). Pleiotropic effects of *Pasteurella multocida* toxin are mediated by $G_q$-dependent and -independent mechanisms. *J. Biol. Chem*. **276,** 3840–3845.

CHAPTER

# 10

# Bacterial toxins and mitochondria

*Antoine Galmiche and Patrice Boquet*

## INTRODUCTION

Although mitochondria have been known for a long time to be important organelles in the energetics of eukaryotic cells, their contribution to some aspects of cell regulation was only discovered during the last decade. In particular, the observation that mitochondria play a critical role in programmed cell death initiated a new interest in their study. During the past five years, a number of reports have documented that some protein toxins and virulence factors produced and secreted by bacteria can target mitochondria of their host cell, shedding new light on the interaction between bacteria and eukaryotic cells. Here, we'll discuss the structure and functions of mitochondria, and we'll review the current state of knowledge regarding bacterial toxins and factors that act on mitochondria.

## STRUCTURE AND BIOGENESIS OF MITOCHONDRIA

Mitochondria are membrane organelles present in all animal cells. Although mitochondria are usually described as rod- or balloon-shaped organelles, their morphological appearance can vary catly from one cell type to the other. Modern imagery techniques showed that, rather than being present as isolated organelles in the cytoplasm, mitochondria form an intracellular reticulum (reviewed in Yaffe *et al*., 1999). They are formed by two membranes, a mitochondrial outer membrane (MOM) and a mitochondrial inner membrane (MIM), containing numerous inner folds. Both membranes delineate an intermembrane space and the mitochondrial matrix. The infoldings of the MIM delineate the mitochondrial cristae, which can be visualized by electron microscopy as tubules and vesicles, in the matrix or in continuity with the intermembrane space. The functional consequences of this sophisticated organization are currently poorly understood.

Mitochondria are motile structures that associate with the cell cytoskeleton, particularly microtubules (Yaffe *et al*., 1999). Like all other membrane organelles, mitochondria are constantly remodeled by fission and fusion processes (Yaffe, 1999). Mitofusins, the vertebrate homologues of the product of the Drosophila protein *fuzzy onion*, a GTPase located in the outer mitochondrial membrane, mediate mitochondrial fusion. Their action is counterbalanced by Dynamin-related proteins (Drp), which catalyze mitochondrial fission events. Thus, the shape of mitochondria and their topological organization are kept under permanent control.

The two mitochondrial membranes have different physical properties and compositions. The MIM contains large amounts of the lipid cardiolipin, and is virtually impermeable to all solutes. The MIM contains the proteins of the respiratory chain and forms the major barrier between the mitochondrial matrix and the cytosol. It plays a paramount role in the cell energetics and metabolism, according to mechanisms that we will mention later. A voltage gradient (expressed as a transmembrane potential $\Delta\Phi m$, in mV) exists across the MIM. This gradient is an important parameter of mitochondrial physiology. It is commonly determined by measuring the uptake of small lipophilic, positively charged dyes endowed with fluorescence properties. The properties of the MOM are less well understood. The MOM was long assumed to be freely permeable to solutes of small size, thanks to the presence of a protein,

the mitochondrial porin (also known as the *Voltage-Dependent Anion Channel*, or VDAC). This view is now challenged, and recent studies suggest that the MOM forms a regulated barrier between the intermembrane space of mitochondria and the cytosol.

An important particularity of mitochondria is the fact that these organelles possess their own genome, consisting in a circular DNA of approx. 16.5 kb, and residing inside the matrix. This DNA is the most evident stigma of the bacterial origin of mitochondria (Gray *et al*., 1999; Dyall *et al*., 2004). According to the now widely accepted endosymbiotic hypothesis, mitochondria are the remnant of an α-proteobacterium that would have once colonized an early eukaryote. Phylogenetic analysis of the mitochondrial genome revealed that members of the rickettsial subdivision of the α-Proteobacteria, a group of obligate intracellular parasites that includes genera such as *Rickettsia*, *Anaplasma*, and *Ehrlichia*, are the closest known eubacterial relatives of mitochondria (Gray *et al*., 1999; Dyall *et al*., 2004). During evolution, the proteobacteria and the eukaryotic cells probably evolved in a concerted fashion: a dramatic process of gene reduction occurred. Important parts of the mitochondrial genome were lost, and others became integrated into the cell nucleus. During this process, mitochondria became *bona fide* organelles (Gray *et al*., 1999; Dyall *et al*., 2004).

In mammals, mitochondria contain the enzymes required for the transcription and the replication of this genome. In addition, the mitochondrial genome contains 13 mRNAs encoding essential subunits of respiratory complexes. Most mitochondrial proteins are encoded by genes that are carried by the nuclear genome. These proteins are synthesized in the cytosol and imported by a specialized protein import machinery formed by protein complexes present in the outer membrane (TOM, for *Translocase of the Outer Membrane*) and in the inner membrane (TIM, for *Translocase of the Inner Membrane*) (review in Wiedemann *et al*., 2004) (see Figure 10.1).

Although these common principles of mitochondrial physiology apply to all tissues, mitochondria from varied tissues have unique properties, as the analysis of their protein contents reveals (Mootha *et al*., 2003). Furthermore, they are functionally heterogenous in a single cell, a phenomenon called *heteroplasmia*.

## MITOCHONDRIA: ORGANELLES DEDICATED TO THE LIFE AND DEATH OF THE CELL

Mitochondria are essential organelles in the regulation of the cell metabolism and energetics. In normal conditions, they are the main producer of the central cellular energy carrier, adenosine triphosphate (ATP). In the mitochondria, this ATP is regenerated from adenosine diphosphate (ADP) in a process called oxidative phosphorylation (see Figure 10.2). But the impact of mitochondria extends beyond the production of energy. Mitochondria regulate many aspects of cellular signaling and metabolic pathways. For example, mitochondria regulate the Ca2+ metabolism. Mitochondria can eventually be observed in close connections with the endoplasmic reticulum (ER) (Rizzuto *et al*., 1998), an organization that could allow them to accumulate Ca2+. The storage and release of Ca2+ from mitochondria affects both the release of this ion from the ER and its entry across the plasma membrane (Rizzuto *et al*., 2004). Very recently it was reported that the intracellular local calcium concentration controlled mitochondrial movement on microtubules, probably by modulating the activity of a myosin (myosin Va) (Yi *et al*., 2004). Mitochondria also play a role in the metabolism of some amino acids and certain lipids: for example, specialized mitochondria play a key role in steroidogenesis in the cells of the adrenal cortex (Jefcoate, 2002). The metabolism of iron is another important aspect of mitochondrial physiology. Mitochondria are the major site of synthesis of the heme and of iron-sulfur (Fe/S) clusters (Lill *et al*., 1999).

The contribution of mitochondria to the control of cellular metabolism has been known for a relatively long time, but a new interest in this organelle came only recently with the observation of its key role in the regulation of apoptosis (reviewed in Kroemer and Reed, 2000). Apoptosis, and by extension most forms of cell death, were found to be tightly programmed events. In many circumstances, apoptosis was observed to be initiated by the release of cytochrome c from the mitochondria into the cytosol (Liu *et al*., 1996). Following this initial seminal observation, it was observed that mitochondria host many other factors whose release into the cytosol can induce apoptosis: beside cytochrome c, these other factors are the flavoprotein AIF, Smac/Diablo, the serine-protease HtrA2/Omi, and a nuclease, endonuclease G (reviewed in Newmeyer and Ferguson-Miller, 2003) (see Figure 10.3). Whereas cytochrome c is normally only present in the mitochondrial intermembrane space, its cytosolic release initiates the assembly of a large protein complex called the *apoptosome*. This complex is responsible for the activation of apoptotic proteases of the caspase family, thereby resulting in the execution of the apoptotic program and the appearance of the typical morphological changes that characterize apoptosis. An increased permeability of the MOM therefore seems to be the main event in the course of apoptosis induction.

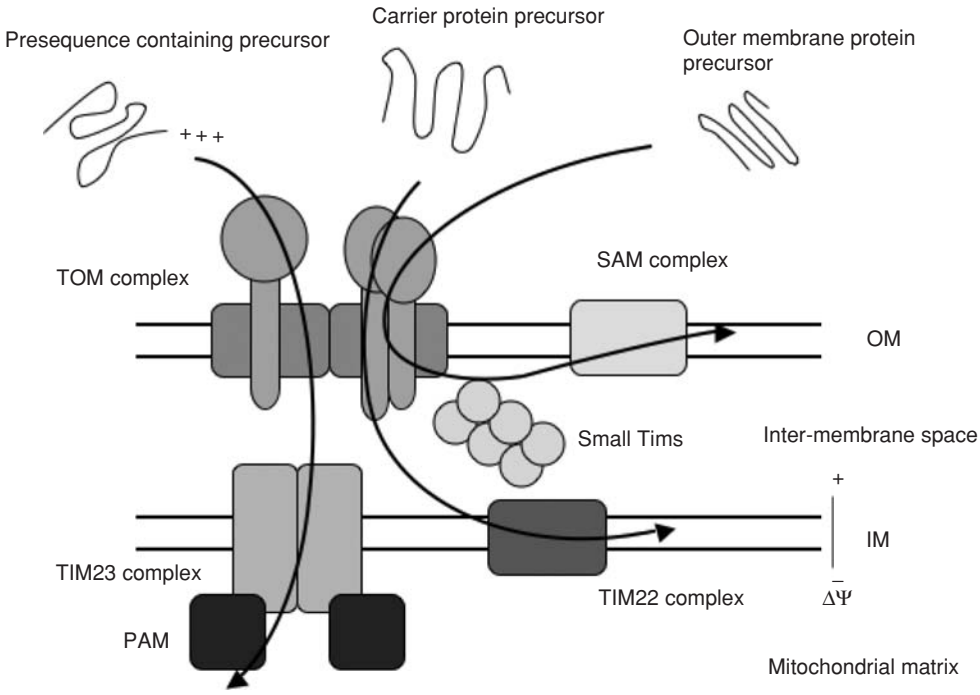



**FIGURE 10.1** Protein import into mitochondria (adapted from Wiedemann *et al*., 2004).

Most mitochondrial proteins are synthesized in the cytosol of the cell before their import into mitochondria. These proteins are often synthesized as precursors containing an amino-terminal signal sequence, usually composed of 20 to 60 residues forming an amphipathic α-helix. Other mitochondrial proteins contain internal targeting signals (in particular, this is the case for hydrophobic precursor proteins destined to the inner membrane, like the ADP/ATP carrier). Cytoplasmic chaperones guide precursor proteins to receptors on the surface of mitochondria. There, preproteins encounter the mitochondrial protein import machinery.

The mitochondrial protein import machinery is formed by protein complexes present in the outer membrane (TOM, for *Translocase of the Outer Membrane*) and in the inner membrane (TIM, for *Translocase of the Inner Membrane*). The TOM complex is the central entry gate for practically all mitochondrial proteins (Wiedemann *et al*., 2004). It includes receptors for the mitochondrial presequence (proteins Tom20 and Tom22) or for proteins with internal targeting information (Tom70), and a protein conducting channel formed by the protein Tom40.

Upon passing through the TOM complex, the precursor proteins can follow one of three major pathways (Wiedemann *et al*., 2004): i) the proteins of the MOM, like the mitochondrial porin (also called VDAC, *Voltage Dependent Anion Channel*), use a protein complex called the SAM (*Sorting and Assembly Machinery*) for their folding and membrane insertion. ii) the preproteins that contain a presequence are transferred to the TIM complex. The preprotein-translocase Tim23, in cooperation with the matrix heat shock protein mtHsp70 actively imports the protein inside the mitochondrial matrix. iii) the preproteins that contain internal targeting signals, like the ADP/ATP carrier, follow a different pathway for their insertion. The TIM22 complex, with the aid of the small Tim proteins, is responsible for insertion of proteins with internal targeting signals into the inner membrane.

As we mentioned earlier, the MOM is normally only permeable to solutes of small size (reviewed in Kroemer and Reed, 2000). How apoptogenic stimuli are converted into changes in the permeability of the MOM is still controversial, but different models have been proposed to account for these changes: they either implicate the formation of pores, the complete rupture of the MOM, or even mitochondrial fission events (Newmeyer and Ferguson-Miller, 2003). Depending on the nature of the apoptotic stimuli that the cell receives, these events might happen in a separate or sequential fashion through mechanisms that are yet unclear. The proteins of the Bcl-2 family play a role in the regulation of these events (reviewed in Cory and Adams, 2002). In mammals, there are at least 20 members of this family of proteins. These proteins share at least one conserved Bcl-2 homology (BH) domain among the four that Bcl-2 contains (BH1–BH4). Members of the Bcl-2 family can be classified into different subtypes, the most evident classification being based on their opposing functions, either as pro-survival or pro-apoptotic member.

i) The pro-survival family. Bcl-2 and Bcl-XL are the best-characterized members of this family. Bcl-XL is predominantly located in the MOM, whereas Bcl-2 has a broader membrane distribution, including the ER and nuclear envelope (Kaufman *et al*., 2003). The members of the pro-survival family of Bcl-2 proteins are able to directly bind and counteract the effect of the pro-apoptotic family members.

ii) The pro-apoptotic family. This family can be further separated into a BH3-only subfamily (Bad, Bid, Bim, Bmf, Noxa, Puma) and the Bax subfamily (Bax

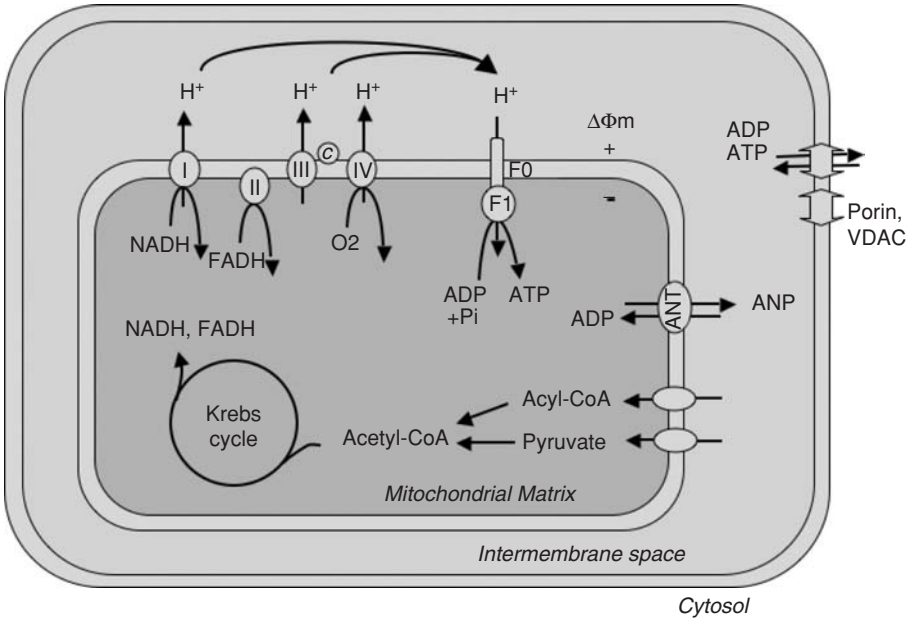



**FIGURE 10.2** Respiration and ATP synthesis in mitochondria

The mitochondria regenerate the cell energy by a process known as *oxidative phosphorylation*. Mitochondria use pyruvate, the end product of glycolysis, and fatty acids to regenerate energy. Pyruvate and acyl chains are actively imported into the mitochondrial matrix. There, they are metabolized into acetyl-CoA and oxidized through a cycle of reactions known as the *tricarboxylic acid cycle*, or Krebs cycle. The high-energy electrons that are extracted during these reactions are transferred to the carriers NAD (Nicotinamide Adenosine Dinucleotide) and FAD (Flavin Adenosine Dinucleotide). The reduced factors NADH and FADH serve as shuttles for these high-energy electrons: they transfer them to the respiratory chain. The mitochondrial respiratory chain is composed of four large enzyme complexes embedded in the inner membrane of the mitochondria. These complexes are the NADH-CoQ reductase (complex I), the succinate-CoQ reductase (complex II), ubiquinol-cytochrome *c* reductase (complex III), and the cytochrome c oxidase (complex IV). The respiratory complexes transfer electrons to each other, and finally to their final acceptor, the oxygen molecule. The coupling of electron transfer to ATP synthesis is indirect, via the constitution of aNH+ electrochemical gradient: the transfer of electrons along the respiratory chain is coupled to the expulsion of protons out of the mitochondrial matrix, across the inner membrane. In this way, respiration creates both a membrane potential ($\Delta\Phi m$) and a pH gradient ($\Delta pH$). These pH and electrical gradients form a proton motive force that the mitochondria uses to synthesize ATP. An enzyme, the F0F1-ATP synthase (also called complex V of the respiratory chain), couples the reentry of protons into the mitochondrial matrix with the chemical conversion of ADP into ATP. This ATP synthase is composed of a F0 portion, crossing the MIM, and connected to a globular, F1 portion, in the mitochondrial matrix. Proton reentry into the matrix, through the associated F0 subunit of ATP synthase induces the rotation of the F1 subunit, and this rotation is coupled to the synthesis of ATP. The F1 portion therefore functions as a genuine rotary molecular motor (Itoh *et al*., 2004; Diez *et al*., 2004). Following its mitochondrial synthesis, ATP is transported across the MIM by the protein ANT (Adenine Nucleotide Translocator). The porin, or VDAC, transports ATP across the MOM.

and Bak) (reviewed in Cory and Adams, 2002). Genetic evidence supports the role of the Bax proteins subfamily as core components of the apoptotic machinery; indeed, Bax/Bak double knock-out cells are resistant to most apoptotic stimuli (Wei *et al*., 2001). On the other hand, the members of the BH3-only subfamily function upstream of Bax/Bak (Zong *et al*., 2001; Cheng *et al*., 2001). A current hypothesis holds that the BH3-only proteins constitute cellular sentinels, and that they are able to activate the Bax/Bak system in response to a variety of death-inducing stimuli. In a living cell, they are normally kept in check by different mechanisms, including sequestration at the subcellular level (interaction with the cell cytoskeleton in the case of Bim and Bmf), post-translational modifications (phosphorylation in the case of Bad, proteolytic processing in the case of Bid), or regulation at the transcriptional level (Noxa and Puma) (review in Cory and Adams, 2002). During apoptosis, protein efflux from mitochondria might be a consequence of local reorganization of the lipids in the MOM, and this process could be driven by the pro-apoptotic protein Bax. Kuwana *et al*. (2002) have reported the use of a cell-free assay using purified components to reconstitute the permeabilization of the MOM by the protein Bax *in vitro*; in this system, Bax added to liposomes mimicking the composition of the MOM rendered them permeable to large-molecular-weight dextran molecules. This process occurred in the

absence of any intrinsic mitochondrial proteins, but required the presence of the mitochondrial lipid cardiolipin (Kuwana *et al*., 2002). In addition to the reorganization of the lipids of mitochondrial membranes, the proteins of the Bcl-2 family might contribute to apoptosis through the changes of mitochondrial shape, especially the mitochondrial fragmentation that are noticed during apoptosis (Karbowski *et al*., 2002, 2004).

Other events take place in the mitochondria of dying cells. A phenomenon known as the *mitochondria-permeability transition* (MPT) is often observed. It is caused by the opening of a pore formed at points of contact of the MOM with the MIM. This pore results in a drop in mitochondrial inner membrane polarity and in the swelling of the mitochondrial matrix. Although its exact role and its molecular composition remain unclear (Kokoszka *et al*., 2004), this pore could cause the complete rupture of the MOM and its non-selective permeabilization (review in Kroemer and Reed, 2000).

Mitochondria are also an important source of reactive oxygen species (ROS). This aspect is worth pointing out because the production of ROS could in some instances participate in cell death. In the normal setting, oxydative phosphorylation produces limited amounts of ROS. An increased production of ROS is often observed at an advanced stage of the apoptotic execution, as a consequence of the cleavage of components of the respiratory chain by caspases (Ricci *et al*., 2004). Clearly, many different events take place in the mitochondria of dying cells, and it is not currently possible to establish a complete causality between them.

## BACTERIAL TOXINS AND VIRULENCE FACTORS TARGETING MITOCHONDRIA

Following a couple of initial reports of bacterial toxins and virulence factors targeting mitochondria, there has been a surge in the number of similar observations. In Table 10.1, we have attempted to list the currently known toxin proteins for which mitochondrial targeting properties were established. This list is not closed, and it is likely to expand as more investigators

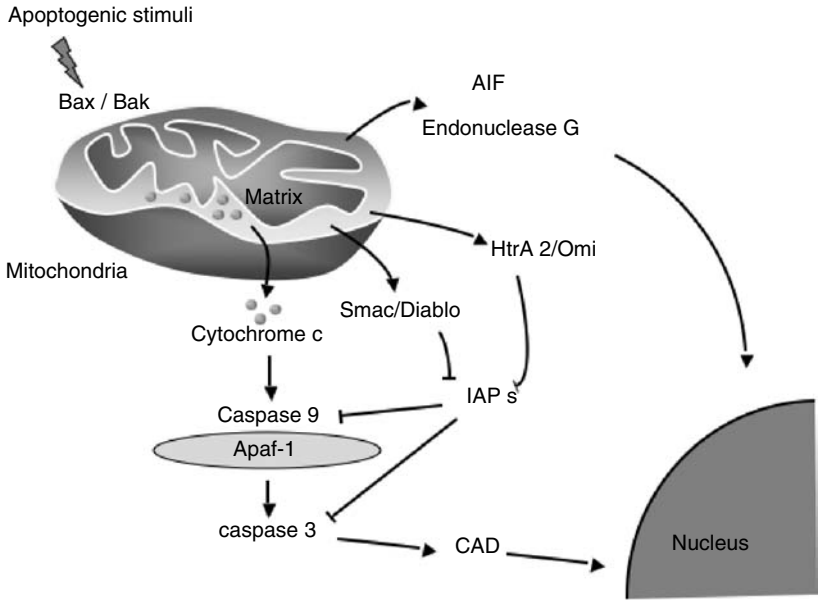



**FIGURE 10.3** Mitochondria and apoptosis

An increased permeability of the MOM occurs early in the course of apoptosis. Apoptogenic stimuli could induce this permeabilization of the MOM through the formation of pores in the MOM, or through its complete rupture. Multiple proteins of the intermembrane space are released into the cytosol. Cytochrome c triggers the formation of the apoptosome, a large protein complex that activates the initiator Caspase-9. Other proteins released from the intermembrane space also contribute to Caspase activation: the Serine-protease HtrA2/Omi and the factor Smac/Diablo that relieve the inhibitory effect of a family of anti-apoptotic factors: the IAPs, that are normally present in the cytosol. The activated Caspase cascade causes the cleavage of multiple cellular substrates. The protein CAD (Caspase-Activated DNAse) is activated and translocates to the nucleus, resulting in the characteristic oligonucleosomal DNA fragmentation observed in apoptotic cells. Other mitochondrial factors can initiate apoptosis through Caspase-independent mechanisms: AIF and endonuclease G can directly translocate from the mitochondria to the nucleus.

now become aware of the existence of mitochondrial targeting by bacterial pathogens. At this point, however, some first conclusions can be drawn.

i) Toxins and virulence factors targeting mitochondria are produced by many microbes. In addition, a single microbe produces several toxins or virulence factors that can act on mitochondria (for example, EPECs produce at least three factors: Tir, MAP, and EspF, that can eventually target mitochondria). Targeting mitochondria seems to be a common event in the bacterial world.
ii) Although the mitochondrial tropism of some of the factors listed in Table 10.1 is now well accepted, for none of these factors do we have a complete understanding of the mode of action inside mitochondria. During our recent investigation of the mode of action of the vacuolating cytotoxin VacA produced by the bacteria *Helicobacter pylori*, we observed that VacA is localized to the MIM following its mitochondrial import (Galmiche *et al*., 2000). There, it is currently unclear whether VacA can form an ion-conducting channel or carry an enzymatic activity (Whillhite and Blanke, 2004). On the other hand, the factor PorB produced by *Neisseria gonorrhoae* inserts into the MOM of infected cells (Müller *et al*., 2002). Although both VacA and PorB are apparently able to induce the apoptosis of infected cells, the mechanisms involved might be quite different.
iii) Another point that remains unclear is the *in vivo* role played by mitochondrial targeting. The production of mitochondrially-acting toxins does not evidently correlate with the microbial lifestyle or the type of infection produced. Considering the many roles played by mitochondria in the cell metabolism, in cell death and regulation, various microbes might exploit mitochondria for different purposes. Mitochondrially-acting toxins of bacterial pathogens are probably "multipurpose tools." It is likely that these purposes will need to be investigated both in an infection- and cell type-specific fashion. Although many reports show that apoptosis regulation is an important consequence of mitochondrial targeting, it is likely that bacterial pathogens could get other benefits from mitochondrially-acting toxins (i.e., inhibition of cells of the immune system, mobilization of the iron from eukaryotic cells). This remains however to to be established.
iv) It is currently unclear how these various toxins and virulence factors reach mitochondria. AB toxins and virulence factors that are injected into cytosol via needle-like, type III secretion (TTSS) (Hueck *et al*., 1998) could gain access to mitochondria after a cytosolic transit step. Alternatively, vesicle-mediated traffic might exist between cellular plasma/endosomes membranes and the mitochondria (Zhang *et al*., 2004).

In the following paragraphs, we'll briefly describe toxins and virulence factors recently documented to target mitochondria.

## The *Neisseriae gonorrhoeae* and *Nesseriae meningitidis* porins PorB

*Neisseriae meningitidis* and *Neisseria gonorrhoeae*, two Gram-negative bacteria, are responsible respectively for meningitis and the sexually transmitted disease gonorrhea. *Neisseria* adhere to and invade epithelial and endothelial cells. Different virulence factors modulate the interactions and cross-talk of these bacteria with host cells, among these type IV pili, Opa proteins, and porins. Meningococcal porin B (MW: 33 kDa) and gonococcal porin B (37 kDa) are β-barrel-structures proteins sharing 70% amino acid sequence homology. In the outer bacterial membrane, they assemble as trimers and form membrane channels implicated in the transport of ions and solutes.

**TABLE 10.1** Bacterial toxins and virulence factors targeting mitochondria

| | | |
|---|---|---|
| PorB | *Neisseria gonorrhae* | (Müller *et al*., 2000, 2002) |
| PorB | *Neisseria meningitidis* | (Massari *et al*., 2000, 2003) |
| VacA | *Helicobacter pylori* | (Galmiche *et al*., 2000; Whillhite and Blanke, 2004) |
| Map | Enteropathogenic *Escherichia coli* (EPEC) | (Kenny and Japson, 2000) |
| Tir | EPEC | (Malish *et al*., 2003) |
| EspF | EPEC | (Nougayrede and Donnenberg, 2004; Nagai *et al*., 2004) |
| Alpha-toxin | *Staphylococcus aureus* | (Bantel *et al*., 2001) |
| SipB | *Salmonella* | (Hernandez *et al*., 2003) |
| Toxin A | *Clostridium difficile* | (He *et al*., 2000, 2002) |
| Toxin B | *Clostridium difficile* | (Fiorentini and Matarese, 2004) |
| Lethal toxin | *Clostridium Sordellii* | (Petit *et al*., 2003) |

The *N. gonorrhoeae* PorB has been shown to induce cell apoptosis during *N. gonorrhoeae* infection of epithelial cells and macrophages or when added as a purified protein to cultured epithelial cells (Müller *et al*., 1999). During infection, when the bacteria is tightly attached on the host cell surface, PorB translocates from the bacterial outer membrane to the cell plasma membrane (Weel and van Putten, 1991). There, it forms voltage-dependent channels regulated in their ion gating activity and selectivity by ATP and GTP nucleotides (Rudel *et al*., 1996). Properties of *N. Gonorrhoeae* PorB resemble thus the VDAC (Rudel *et al*., 1996). This prompted Müller *et al*. (2000) to examine whether apoptosis induced by PorB was due to a mitochondrial activity of the bacterial porin. Treatment of Jurkat T cells, monocytes, or epithelial cells with purified PorB induced a loss of the $\Delta\Phi$m and the release of cytochrome c from mitochondria (Müller *et al*., 2000). Expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-XL, prevented PorB-induced cytochrome c release and apoptosis, showing that gonoccocal PorB induces apoptosis through the intrinsic pathway (Müller *et al*., 2000). Targeting of PorB to mitochondria was subsequently demonstrated by cell fractionation and immunofluorescence studies (Müller *et al*., 2000). The cellular pathway that PorB uses to be transported from the bacterial surface to mitochondria is unknown. The deorganization of actin filaments or microtubules does not prevent PorB from reaching mitochondria, leading to the conclusion that endocytosis and endosome trafficking are probably not implicated in the transport of the porin from the cell surface to mitochondria (Müller *et al*., 2000). No signal sequence or linear determinant responsible for mitochondrial import could be identified in the sequence of PorB (Müller *et al*, 2002). Mitochondrial insertion of PorB into the MOM was found to require the protein import machinery of this organelle, including the Tom20 receptor and the general import pore (Müller *et al*., 2002).

How does *N. gonorrhoeae* PorB provoke cytochrome c release from mitochondria? Following its intracellular expression into HeLa cells, it was observed that *N. gonorrhoeae* PorB localizes to the MOM and forms oligomers (Müller *et al*., 2002). These authors reported that the gonococcal PorB could interact with the ANT, but not with the VDAC (Müller *et al*., 2002). As a consequence, they proposed that PorB might induce apoptosis through the induction of the MPT.

*Neisseria meningitidis* PorB, like *N. gonorrhoeae* PorB, was also found to target mitochondria (Massari *et al*., 2000; Massari *et al*., 2003a). In contrast to its homologue in *N. gonorrhoeae*, PorB produced in *N. meningitidis* was not toxic for cells. No apoptosis could be detected when HeLa or Jurkat cells were exposed for 24 h to doses as high as 100 μg/ml of purified *N. meningitidis* PorB (Massari *et al*., 2000; Massari *et al*., 2003a). No modification of the $\Delta\Phi$m, swelling of mitochondria, or release of cytochrome c could be observed in these conditions (Massari *et al*., 2000; Massari *et al*., 2003a). Moreover, *N. meningitidis* PorB afforded protection, like Bcl-XL or Bcl-2, against a proapoptotic stimulation induced by staurosporine (Massari *et al*., 2000; Massari *et al* 2003a). In cells treated with *N. meningitidis* PorB, the porin was shown to co-immunoprecipitate with the mitochondrial VDAC, but not ANT (Massari *et al*., 2000). From these data, it was hypothetized that the *N. meningitidis* PorB, by interacting with VDAC, might this time prevent the occurrence of the MPT, perhaps in a fashion similar to Bcl-2 or Bcl-XL, resulting in an increased cell survival (Massari *et al*., 2000; Massari *et al*., 2003b).

How can two highly homologous proteins induce opposing effects on cell apoptosis? It has been argued that, rather than a difference between the structure of the two porins, the methods used by Müller *et al*. (2000) or Massari *et al*. (2000) to purify PorB (in particular the detergent concentration) or to perform the intoxication were probably responsible for the opposing porin activities on mitochondria (Massari *et al*., 2000; Massari *et al*., 2003b). In view of these conflicting results, it is currently difficult to propose an *in vivo* role for *Neisserial* PorB action on host mitochondria.

## The *Helicobacter pylori* VacA cytotoxin

*Helicobacter pylori* is a Gram-negative bacterium that infects 50% of the world population. *H. pylori* is determinant in the pathogenesis of peptic ulcer, chronic gastritis, gastric carcinoma, and the lymphoma deriving from the mucosa-associated lymphoid tissue (reviewed in Blaser and Atherthon, 2004). Several virulence factors are associated with *H. pylori,* in particular the vacuolating cytotoxin VacA. We will only briefly describe here this molecule, since this cytotoxin is reviewed in detail in another chapter of this book.

VacA has initially been identified by its ability to induce large vacuoles in cultured cells (reviewed in Papini *et al*., 2001). Cell vacuolation by VacA requires the presence of weak bases, such as ammonium chloride, and develops selectively from late endosomes (Papini *et al*., 2001). In its mature form, VacA has a molecular weight of 87 kDa. Once released from the bacteria, the cytotoxin is cleaved into two subunits of 34 kDa (the N-terminal fragment p34) and 58 kDa (the C-terminal fragment p58) that remain associated by non-covalent interactions (Papini *et al*., 2001). VacA binds host cells on one or several receptor(s) putatively identified as receptor tyrosine phosphatase α (RPTPα) (Yahiro *et al*., 2003)

and VacA localizes in lipid rafts (Ricci *et al*., 2000; Schraw *et al*., 2001; Patel *et al*., 2001; Gauthier *et al*., 2004). Full activity of VacA requires GPI-anchored proteins (Ricci *et al*., 2000; Kuo and Wang, 2003; Gauthier *et al*., 2004). In the membrane bilayer, the cytotoxin hexamerises and forms channels that are selective for anions on the cell surface (Papini *et al*., 2001). These channels are pinocytosed by an actin-dependent mechanism, which does not require clathrin or dynamin (Ricci *et al*., 2000; Gauthier *et al*., 2004). Once in the late endosomes, VacA channels increase the permeability of this membrane organelle to anions. This facilitates the function of the V-ATPase, the proton pump that is responsible for the acidification of intracellular compartments. This leads to an accumulation of osmotically active species that results in the swelling of these compartments (Gauthier *et al*., 2004). Beside its vacuolating activity, VacA also induces apoptosis in cultured cells (Kuck *et al*., 2001; Cover *et al*., 2003). The mechanism by which the cytotoxin induces cell apoptosis was first shown by expressing the p34 or the p58 cytotoxin fragments fused with the Green Fluorescent Protein (GFP) into epithelial cells: using this strategy, we observed that GFP-p34, but not GFP-p58, colocalized with mitochondria (Galmiche *et al*., 2000) (see Figure 10.4). It was subsequently demonstrated that VacA and its p34 fragment (but not p58) were imported *in vitro* into isolated mitochondria (Galmiche *et al*., 2000). It was shown that VacA and the p34 fragment were imported into the MIM or the matrix compartment of mitochondria (Galmiche *et al*., 2000). At the same time, we noticed that the intracellular expression of the p34 fragment or the full length VacA in HEp-2 cells could induce cytochrome c release and a subsequent activation of the effector caspase-3 (Galmiche *et al*., 2000). HEp-2 cells incubated with the purified native toxin for a prolonged period of time (24 h) also exhibited a release of cytochrome c, indicating that VacA is indeed targeting mitochondria when it is applied outside of cells (Galmiche *et al*., 2000). From these observations, it was concluded that VacA resembles an AB toxin in which the A (p34) moiety target mitochondria, whereas the B subunit (p58) allows the binding of the cytotoxin to host cells and the penetration of the active subunit into the cytosol. Our observations clarified a previous report indicating that VacA could induce mitochondrial damages in human gastric cells (producing a drop in $\Delta\Phi$m, an inhibition of the cell oxygen consumption, and an ATP depletion) (Kimura *et al*., 1999). These data were subsequently confirmed and extended by other groups showing (i) that incubation of purified VacA with HeLa or gastric AZ-521 cells induces the release of cytochrome c into the cytosol (Willhite *et al*., 2003; Nakayama *et al*., 2004), (ii) that VacA incubated with gastric AZ-21 or Hela cells induces a decrease in $\Delta\Phi$m (Nakayama *et al*., 2004; Willhite and Blanke, 2004), and (iii) that fluorescently labeled VacA applied to Hela cells localizes to mitochondria (Willhite and Blanke, 2004). Like *Neisseriae* PorB, VacA does not contain any linear targeting sequence responsible for mitochondrial targeting; residues spread over the all p34 moiety are important (Galmiche *et al*., 2000).

We are now focusing our investigation on the mode of action of VacA inside the mitochondria. Two modes of action have been proposed. A first model holds that the ability of VacA to form anionic channels in lipidic membranes is required for the mitochondrial cytotoxin activity (Willhite *et al*., 2003; Willhite and Blanke, 2004). Such an anionic channel, assembled in the MIM, would result in the collapse of the $\Delta\Phi$m and the release of cytochrome c. This model is supported by the protective effect of pharmacological blockers of VacA-dependent chloride transport on the mitochondrial toxicity of VacA (Willhite and Blanke, 2004). In addition, a mutant of VacA defective in its anionic channel

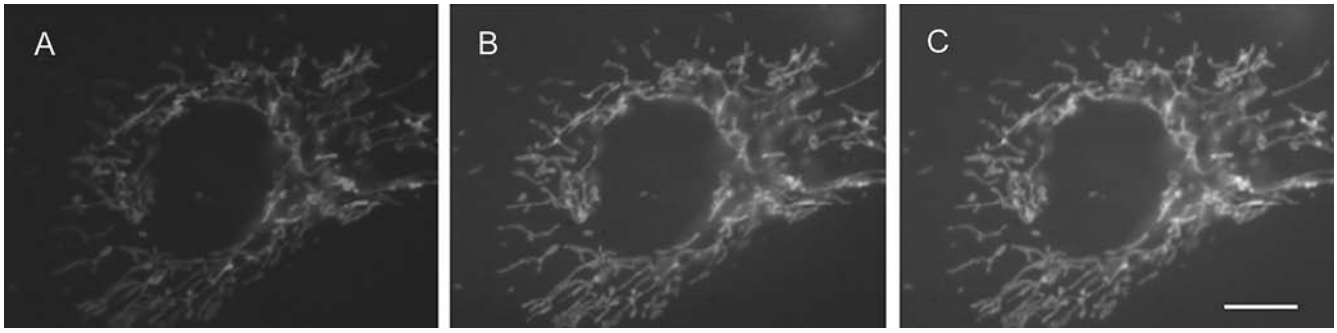



**FIGURE 10.4.** The vacuolating cytotoxin VacA of *Helicobacter pylori* targets mitochondria

Human umbilical vein epithelial cells (HUVEC) were co-transfected with the construct DsRed-mito, allowing the live visualization of mitochondria (panel A), and the amino-terminal part of the cytotoxin VacA of *Helicobacter pylori*, the protein p34 fused to GFP (panel B). A nearly total overlap of the two stainings was observed (see the overlay presented in panel C). Bar : 10 µm.

formation because of a 22 amino acid residues deletion (Δ6-27 VacA) is still targeted to mitochondria, albeit unable to modify the ΔΦm (Vinion-Dubiel *et al*., 1999; Willhite and Blanke, 2004). The model of a VacA channel-mediating toxicity is however challenged by the observation that VacA-induced vacuolation and effects on mitochondria are dissociable events: the intracellular expression of p34 induces cytochrome c release and cell death without any vacuolation (Galmiche *et al*., 2000). In fact, the p34, albeit unable to oligomerize and thus to form channels (Papini *et al*., 2001), has the same toxic activity as the whole VacA molecule on mitochondria (Galmiche *et al*., 2000). While it remains possible that p34 alone could oligomerize and create a channel in the MIM, we would like to propose an alternative model for the mode of action of VacA, where p34 might be endowed with an enzymatic activity that modifies one or several mitochondrial target molecule(s).

An interesting issue is the mechanism by which VacA, upon binding to its cell surface receptor, is transferred to mitochondria. We proposed the following model. The ability of VacA to induce the swelling of endosomes might have been exploited to rupture these compartments, thereby releasing the cytotoxin or the p34 fragment in the cytosol and allowing it to reach mitochondria. That model is derived from the cellular mechanism by which DNAs can be transfected into cells using charged polyplexes molecules, such as polyethylene imines (PEIs). PEIs are known to induce endosome swelling by provoking, like VacA, an influx of chloride ions into these compartments and are used as drugs to increase DNA transfections (Sonawane *et al*. 2003). Swelling is followed by the rupture of a small population of endosomes and the release of transfectant DNAs (Sonawane *et al*. 2003). That VacA uses such a mechanism might be supported by the observation that apoptosis of gastric AGS cells induced by VacA is promoted by the weak base ammonium chloride, required for VacA-induced late endosome swelling (Cover *et al*., 2003).

We have recently suggested that VacA, being produced by *H. pylori* in the gastric mucosa, could target the cells of the epithelium, and, in particular, parietal cells. This cell type produces chlorhydric acid, and is one of the cell types richest in mitochondria in the organism. We have proposed that VacA intoxication of these cells could reduce the gastric acid secretion and help *Helicobacter pylori* colonize the gastric mucosa, either through apoptosis induction or by "de-energizing" these cells (Boquet *et al*., 2003). In the long term, apoptosis induction inside the gastric epithelium could play a distinct pathogenic role. Continuous apoptosis induction might promote the appearance of a pre-malignant state, known as *atrophic gastritis* (Shirin and Moss, 1998).

## Enteropathogenic *Escherichia coli* (EPEC) Map, Tir and Espf virulence factors

Enteropathogenic *Escherichia coli* is a major cause of diarrhea in young children in developing countries (reviewed in Nataro and Kaper, 1998). EPEC infect the intestinal epithelium. While these bacteria remain extracellular, they induce a characteristic modification of the cell cytoskeleton that causes the formation of a pedestal made of filamentous actin. At this level, the bacteria strongly adhere to the eukaryotic host via the afimbrial adhesin intimin. EPEC induce host epithelial cell apoptosis (Abul-Milh *et al*., 2001). To produce these effects, EPEC inject, by a TTSS, the protein Tir (the receptor for intimin but also an inducer of actin polymerization) (Kenny *et al*., 1997). Other bacterial proteins appear to be co-injected by TTSS into the host cell together with Tir including, Map and EspF. Map, Tir, and EspF have been shown to be targeted to mitochondria (Kenny and Jepson, 2000; Malish *et al*., 2003; Nougayrede and Donnenberg, 2004; Nagai *et al*., 2004).

The mitochondrial associated protein (Map) (also known as *Orf19*) contains an N-terminal mitochondrial amino acid sequence and targets mitochondria where it induces a drop of the ΔΦm (Kenny and Jepson, 2000). However, it is yet not clear whether Map induces cell apoptosis (Kenny and Jepson, 2000). Independently of its activity on mitochondria, Map also interferes with the actin cytoskeleton via the GTPase Cdc42, and it plays a role with Tir in the bacterial cell invasion (Jepson *et al*., 2003).

The intimin receptor Tir when expressed by transfection into cells localizes to mitochondria and induces apoptosis of transfected cells (Malish *et al*., 2003). However, localization of Tir into mitochondria of EPEC-infected cells has not been observed (Malish *et al*., 2003). Tir, being the receptor for intimin, contains a membrane-spanning domain, which might also incorporate, upon expression into cells, into the mitochondrial MOM.

EspF has been shown to induce intestinal epithelium tight junctions opening, contributing to diarrhea caused by EPEC (McNamara *et al*., 2001). EspF contains a consensus mitochondrial targeting N-terminal sequence (Nougayrede and Donnenberg, 2004; Nagai *et al*., 2004) and colocalizes with mitochondria in EPEC infected cells (Nougayrede and Donnenberg, 2004; Nagai *et al*., 2004). EspF was shown to modify the ΔΦm, to induce cytochrome c leading to activation of the executioner caspase 3 causing cell death (Nougayrede and Donnenberg, 2004; Nagai *et al*., 2004). Importantly, the

ability of EspF to target mitochondria was linked to the bacterial pathogenesis and colonization in an animal model of infection (Nagai *et al*., 2004).

## *Staphylococcus aureus* α-toxin

*Staphylococcus aureus* is a frequent pathogen for human and animals and represents an important concern in nosocomial infections. Patients with severe *S. aureus* sepsis and undergoing multiple organ failure exhibit lymphocyte apoptosis, which might account for the depletion of immune cells favoring bacterial spread. *S. aureus* secretes many virulence factors among these pore-forming toxins (for more details see chapters on pore-forming toxins in this book). It was recently shown that the pore-forming α-toxin of *S. aureus* is a major mediator of lymphocyte apoptosis induced by this bacterium (Bantel *et al*., 2001). The α-toxin induced the release of cytochrome c from Jurkat T cells or primary T lymphocytes together with activation of the executionner caspase 3 (Bantel *et al*., 2001). Release of cytochrome c could also be induced directly on isolated mitochondria by the toxin (Bantel *et al*., 2001). Furthermore, it was demonstrated that the release of cytochrome c was under the control of the anti-apoptotic factor Bcl-2 (Bantel *et al*., 2001). These observations led to the conclusion that α-toxin acted selectively on the mitochondrial pathway of apoptosis to induce cell death (Bantel *et al*., 2001). However, in this report, it was not demonstrated that the α-toxin localized into mitochondria in toxin-treated cells. These conclusions were subsequently slightly modified by observations showing that primary lymphocytes, in contrast to Jurkat cells, were able to produce TNF-α upon α-toxin incubation (Haslinger *et al*., 2003). TNF-α by stimulating the caspase-8 pathway of apoptosis could induce cell death independently from the mitochondrial pathway (Haslinger *et al*., 2003). In addition, it was shown that *S. aureus* α-toxin could provoke the cell death of Jurkat T or MCF-7 breast carcinoma cells in a necrotic-like manner by a caspase-independent mechanism (Essmann *et al*., 2003). Therefore, it is not yet clear which cell death pathway *S. aureus* α-toxin manipulates during an infectious process *in vivo*.

## *Salmonella* SipB

The facultative intracellular pathogen *Salmonella* triggers programmed cell death in macrophages (reviewed in Hueffer and Galàn, 2004). This programmed cell death is essentially mediated by the protein SipB, a factor that is encoded in the pathogenicity island SPI-1 of *Salmonella* (SPI-1 for *Salmonella* Pathogenicity Island-1). This pathogenicity island encodes a TTSS that secretes SipB and allows it to reach the plasma membrane of the cells that are infected by *Salmonella*.

SipB essentially induces the apoptosis of the host macrophage in a caspase-1 dependent fashion (Hersch *et al*., 1999), but caspase-1-independent mechanisms also exist (Hueffer and Galan, 2004). A recent work suggests that a mitochondrial action of SipB could explain this caspase-1-independent pro-apoptotic effect (Hernandez *et al*., 2003). This conclusion was reached using intracellular expression of SipB in cells that are resistant to caspase-1 activation by *Salmonella* (primary macrophages from caspase-1 knock-out mice and epithelial cells): in the epithelial cell line COS, expression of SipB induced the formation of multilamelar structures that resembled autophagic structures and contained endoplasmic reticulum and mitochondrial markers (Hernandez *et al*., 2003). In caspase-1-deficient macrophages infected with *Salmonella*, SipB localized to mitochondria, and these organelles displayed swelling and disparition of cristae. Finally, an increase in the formation of autophagosomes was noticed, and a form of cell death occurred that could not be prevented by the pharmacological inhibition of the caspases (Hernandez *et al*., 2003). Based on these observations, it was proposed that SipB might target the mitochondria of its host cells and induce cell death with autophagy as an essential morphological feature (Hernandez *et al*., 2003). In this respect, autophagy is a common finding in some forms of caspase-independent cell death (Locksin and Zakeri, 2002).

The molecular details of SipB mode of action on mitochondria are unknown, but Hernandez *et al*. (2003) have extended their conclusions with a model based on SipB ability to promote the membrane fusion of liposomes mimicking the composition of the bacterial and cellular membranes, especially those containing the lipid cardiolipin, an abundant component of mitochondrial membranes (Hayward *et al*., 2000). They proposed that, following its localization to mitochondria, SipB would exert its membrane fusion activity in a fashion reminiscent of the protein mitofusin and induce the appearance of the observed multilamellar structures (Hernandez *et al*., 2003). Although appealing, this model remains largely unproven, and more work will be required to clarify the mitochondrial effect of SipB.

## Large clostridial cytotoxins

The high-molecular-weight cytotoxins (MW 250–300 kDa) produced by the anaerobic bacteria *Clostridium sordelli* (lethal (LT) and hemorragic (HT) toxins), *C. difficile* (toxin A (CdtA) and B (CdtB)) and *C. novyi*

(α toxin) have been grouped under the term of large clostridial cytotoxins (LCCs) (Eichel-Streiber *et al*., 1996). LCCs are described in detail in other chapters of this book, and we will outline only briefly their main features. All these cytotoxins affect the organization of the host cell actin cytoskeleton (reviewed in Eichel-Streiber *et al*., 1996). The LCCs, CdtA, CdtB, and HT all exhibit glucosyltransferase activity and modify small GTPases of the Rho family on threonine 35/37 of their effector loop (reviewed in Boquet and Lemichez, 2003b). The *C. sordellii* LT toxin isolated from strain IP-82 glucosylates the GTPases Ras, Rap, and Ral (reviewed in Boquet and Lemichez, 2003b).

CdtB was first shown to induce apoptosis of intestinal crypt cells (Fiorentini *et al*., 1998). Later, CdtA was described to localize to mitochondria and to induce early mitochondrial damages, including cytochrome c release, ATP depletion, reactive oxygen production, and cell apoptosis (He *et al*., 2000, 2002). Interestingly, it was shown that CdtA could induce leakage of cytochrome c from isolated mitochondria (He *et al*., 2000). Effects of CdtA on mitochondria appeared to be independent from the activity of the cytotoxin on the actin cytoskeleton via the Rho GTPases (He *et al*., 2000). More recently, the LT toxin from *C. sordellii* strain IP82 was shown to localize to mitochondria and to induce cytochrome c release leading to apoptosis by the mitochondrial pathway in HL 60 cells (Petit *et al*., 2003). LT enzymatic activity on small GTPases did not appear to play a role in inducing cell apoptosis (Petit *et al*., 2003). However, the toxin activity on the Ral GTPase to provoke cell death could not be totally excluded (Petit *et al*., 2003). Indeed, LT toxin from *C. sordellii* strain 9048 that exhibits the same spectrum of small GTPases modifications as LT-IP82, except that it does not glucosylate the Ral GTPase, was not able to induce cell death on HL 60 (Petit *et al*., 2003). Interestingly, it has been shown very recently that the 18 N-terminal residues of LT contains a phophatidylserine binding site required for the full glucosylation of the membrane-attached Rac GTPase (Mesmin *et al*., 2004). It might be possible that LT may exhibit also some affinity for lipids of MOM, thereby allowing alteration of the mitochondrial MPT. CdtB is highly homologous to *C. sordellii* LT toxin (88% of homology at the amino acid sequence and both toxins cross-react immunologically). Recently, it was observed that purified or recombinant CdtB added to isolated mitochondria were each able to alter the MIM $\Delta\Phi m$ (Fiorentini and Matarese, 2004). In contrast to *C. sordellii* LT, CdtB does not exhibit an affinity for lipids at the level of its N-terminal amino acids (Mesmin *et al*., 2004). It remains to be demonstrated whether CdtA, CdtB, and LT act on mitochondria by similar or different molecular mechanisms.

## WHY DO BACTERIAL TOXINS AND VIRULENCE FACTORS TARGET MITOCHONDRIA ?

Is mitochondrial targeting an event of frequent occurrence for bacterial proteins, and toxins in particular? A recent computer-based survey of the proteins present in the genome of. *E.coli* showed that up to 5% of the proteins encoded have sequence features that could serve as mitochondrial targeting information (Luccatini *et al*., 2004). An explanation for this frequent "predisposition" is likely to be found in the phylogenetic origin of mitochondria (Dyall *et al*., 2004). The study of the topogenesis of β-barrel membrane proteins of the MOM and the outer membrane of bacteria recently strikingly illustrated one consequence: the conservation of protein transport mechanisms between bacteria and human mitochondria (Paschen *et al*., 2003). In line with this observation, the recent study of the mitochondrial import of *Neisseria* PorB revealed that this porin shares its mitochondrial import pathway with its cousin, the mitochondrial porin/VDAC (Müller *et al*., 2002). A complete discussion of the consequences of this conservation is clearly beyond the scope of this review, but we would like to suggest that, in the context of bacterial pathogenesis, evolution might have favored mitochondrial targeting because of the many similarities that exist between mitochondria and Gram-negative bacteria.

## CONCLUSION

The study of mitochondrial targeting by bacterial virulence factors is a developing field. Because mitochondria are organelles that probably originated from bacteria, evolution could have favored the constitution of virulence factors interfering with these organelles. Studies on mitochondria, first reduced as providers of energy, show that these organelles play important roles in the regulation of cell homeostasis. Regulatory mechanisms of the cellular homeostasis have been constantly exploited by virulence factors of pathogens. Deciphering the activities of those factors will undoubtly lead to the discovery of important new concepts in cell biology.

## ACKNOWLEDGMENTS

We would like to thank Nils Gauthier (INSERM U 627, Nice, France) for his contribution to Fig. 10.4, Carla Fiorentini and Paola Matarese (Istituto Superiore di

Sanità, Roma, Italy) for their communication of unpublished results, and Ulrike Rapp-Galmiche (Max Planck Institut für Infektions, Berlin, Germany) for reviewing the manuscript.

## REFERENCES


Abul-Milh, M., Wu, Y., Lau, B., Lingwood, C.A. and Foster, D.B. (2001). Induction of epithelial cell death including apoptosis by entropathogenic *Escherichia coli* expressing bundle-forming pili. *Infect. Immun*. **69**, 7356–7364.

Bantel, H., Sinha, B., Domschke, W., Peters, G., Schulze-Osthoff, K. and Janicke, R.U. (2001). Alpha-toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J. Cell. Biol*. **155**, 637–48.

Blaser, M.J. and Atherton, M.C. (2004). *Helicobacter pylori* persistence: biology and disease. *J. Clin. Invest*. **113**, 321–333.

Boquet, P., Ricci, V., Galmiche, A. and Gauthier, N. (2003a). Gastric cell apoptosis and *Helicobacter pylori*: has the main function of VacA finally been identified ? *Trends Microbiol*. **11**, 410–3.

Boquet. P. and Lemichez, E. (2003b). Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? *Trends Cell Biol*. **13**, 238–246.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T. and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell*. **8**, 705–711.

Cory, S. and Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2**, 647–656.

Cover, T.L., Krishna, U.S., Israel, D.A. and Peek, R.M. (2003). Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Res*. **63**, 951–957.

Diez, M., Zimmermann, B., Borsch, M., Konig, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekyan, S., Kudryavtsev, V., Seidel, C.A. and Graber, P. (2004). Proton-powered subunit rotation in single membrane-bound F0F1-ATP synthase. *Nat. Struct. Mol. Biol*. **11**, 135–141.

Dyall. S.D., Brown, M.T. and Johnson, P.J. (2004). Ancient invasions: from endosymbionts to organelles. *Science* **304**, 253–257.

Eichel-Streiber, C., Boquet, P., Sauerborn, M. and Thelestam, M., (1996). Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. *Trends in Microbiol*. **4**, 375–382.

Essmann, F., Bantel, H., Totzke, G., Engels, I.H., Sinha, B., Schulze-Osthoff, K. and Janicke, R.U. (2003). *Staphylococcus aureus* alpha-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation. *Cell Death Differ*. **10**, 1260–1272.

Fiorentini, C., Fabbri, A., Falzano, L., Fattorossi, A., Matarese, P., Rivabene, R. and Donelli, G. (1998). *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. *Infect. Immun*. **66**, 2660–2665.

Fiorentini, C. and Matarese, P. (2004). Manuscript in preparation (Personal communication).

Galmiche, A., Rassow, J., Doye, A., Cagnol, S., Chambard, J.C., Contamin, S., de Thillot, V., Just, I., Ricci, V., Solcia, E., Van Obberghen, E., Boquet, P. (2000). The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J*. **19** 6361–6370.

Gauthier, N.C., Ricci, V., Gounon, P., Doye, A., Tauc, M., Poujeol, P. and Boquet, P. (2004). Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the *Helicobacter pylori* vacuolating toxin VacA in HeLa cells. *J. Biol. Chem*. **279**, 9481–9489.

Gray, M.W., Burger, G. and Lang, B.F. (1999). Mitochondrial evolution. *Science* **283**, 1476–1481.

Haslinger, B., Stranfeld, K., Peters, G., Schulze-Osthoff, K. and Sinha, B. (2003). *Staphylococcus aureus* α-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumor necrosis factor-α and the mitochondrial death pathway. *Cell Microbiol*. **5**, 729–741.

Hayward R.D., McGhie E.J. and Koronakis V. (2000). Membrane fusion activity of purified SipB, a *Salmonella* surface protein essential for mammalian cell invasion. *Mol. Microbiol*. **37**, 727–39.

He, D., Hagen, S.J., Pothoulakis, C., Chen, M., Medina, N.D., Warny, M. and Lamont, J.T. (2000). *Clostridium difficile* toxin A causes early damage to mitochondria in cultured cells. *Gastroenterology* **119**, 139–150.

He, D., Sougioultzis, S., Hagen, S., Liu, J., Keates, S., Keates, A.C., Pothoulakis, C. and Lamont, J.T. (2002). *Clostridium difficile* toxin A triggers human colonocyte IL-8 release via mitochondrial oxygen radical generation. *Gastroenterology* **122**, 1048–1057.

Hernandez, L.D., Pypaert, M., Flavell, R.A. and Galàn, J.E. (2003). A *Salmonella* protein causes macrophage cell death by inducing autophagy. *J. Cell Biol*. **163**, 1123–1131.

Hersch, D., Monack, D., Smith, M., Ghori, N., Falkow, S. and Zychlinsky, A. (1999). The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase 1 proc. *Natl. Acad. Sci. USA* **96**, 2396–2401.

Hueck, C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev*. **62**, 379–433.

Hueffer, K. and Galàn, J.E. (2004). *Salmonella*-induced macrophage death: multiple mechanisms, different outcomes. *Cell Microbiol*. **11**, 1019–1025.

Itoh, H., Takahashi, A., Adachi, K., Noji, H., Yasuda, R., Yoshida, M. and Kinosita, K. (2004). Mechanically-driven ATP synthesis by F1-ATPase. *Nature* **427**, 465–468.

Jefcoate, C. (2002). High-flux mitochondrial cholesterol trafficking, a specialized function of the adrenal cortex. *J. Clin. Invest*. **110**, 881–890.

Jepson, M.A., Pellegrin, S., Peto, L., Banbury, D.N., Leard, A.D., Mellor, H. and Kenny, B. (2003). Synergistic roles for map and Tir effector molecules in mediating uptake of enteropathogenic *Escherichia coli* (EPEC) into non-phagocytic cells. *Cell Microbiol*. **5**, 773–783.

Karbowski, M., Lee, Y.J., Gaume, B. Jeong, S.Y., Franck, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C.L and Youle, R.J. (2002). Spatial and temporal association of Bax with mitochondrial fission sites, Drp1 and Mfn2, during apoptosis. *J. Cell Biol*. **159**, 931–938.

Karbowski, M., Arnoult, D., Chen, H., Chan, D.C., Smith, C.L. and Youle, R.J. (2004). Quantitation of mitochondrial dynamics by photolaleling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis. *J. Cell Biol*. **164**, 493–499.

Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R. and Borner, C. (2003). Characterization of the signal that directs Bcl-xL, but not Bcl-2, to the mitochondrial outer membrane. *J. Cell Biol*. **160**, 553–564.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A. and Finlay, B.B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**, 511–520.

Kenny, B. and Jepson, M. (2000). Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cell Microbiol*. **2**, 579–590.

Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hiramaya, T. and Kondo, T. (1999). Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in gastric cells. *Microb. Pathog*. **26**, 45–52.

Kokoszka, J.E., Waymire, K.G., Levy, S.E., Sligh, J.E., Cai, J., Jones, D.P., MacGregor, G.R. and Wallace, D.C. (2004). The ADP/ATP translocator is not essential for the mitochondrial permeability pore. *Nature* **427**, 461–5.

Kroemer, G. and Reed, J.C. (2000). Mitochondrial control of cell death. *Nature Med*. **6**, 513–519.

Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P.H., Stremmel, W. and Rudi, J. (2001). Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. *Infect. Immun*. **69**, 5080–5087.

Kuo, C.H. and Wang, W.C. (2003). Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells. *Biochem. Biophys. Res. Commun*. **303**, 640, 644.

Kuwana, T., Mackey, M.R., Perkins, G., Ellisman, M.H., Latterich, M., Schneiter, R., Green, D.R. and Newmeyer, D.D. (2002). Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331–342.

Lill, R., Diekert, K., Kaut, A., Lange, H., Pelzer, W., Prohl, C. and Kispal, G. (1999). The essential role of mitochondria in the biogenesis of cellular iron-sulfur proteins. *Biol. Chem*. **380**, 1157–1166.

Liu. X., Kim, C.N., Yang, J., Jemmerson, R., and Wang. X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*. **86**, 147–157.

Locksin, R.A. and Zakeri, Z. (2002). Caspase-independent cell deaths. *Curr. Opin. Cell Biol*. **14**, 727–733.

Lucattini, R., Likic, V.A. and Lithgow, T. (2004). Bacterial proteins predisposed for targeting to mitochondria. *Mol. Biol. Evol*. **21**, 652–658.

MacNamara, B.P., Koutsouris, A., O'Connell, C.B., Nougayrede, J.P., Donnenberg, M.S. and Hecht, G. (2001). Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *J. Clin. Invest*. **107**, 621–627.

Malish, H.R., Freeman, N.L., Zurawski, D.V., Chowrashi, P., Ayoob, J.C., Sanger, J.W. and Sanger, J.M. (2003). Potential role of the EPEC translocated intimin receptor (Tir) in host apoptotic events. *Apoptosis* **8**, 179–190.

Massari, P., Ho, Y. and Wetzler, L.M. (2000). *Neisseria meningitidis* porin PorB interacts with mitochondria and protects cells from apoptosis. *Proc. Natl. Acad. Sci. USA* **97**, 9070–9075

Massari, P., King, C.A., Ho, A.Y. and Wetzler L.M. (2003a). Neisserial PorB is translocated to the mitochondria of HeLa cells infected with *Neisseria meningitidis* and protects cells from apoptosis. *Cell Microbiol*. **5**, 99–109.

Massari, P., Ram, S., Mcleod, H. and Wetzler, L.M. (2003b). The role of porins in neisserial pathogenis immunity. *Trends in Microbiol*. **11**, 87–93.

Mesmin, B., Robbe, K., Geny, B., Luton, F., Brandolin, G., Popoff, M.R. and Antonny, B. (2004). A phosphatidylserine-binding site in the cytosolic fragment of Clostridium sordellii lethal toxin facilitates glucosylation of membrane-bound Rac and is required for cytotoxicity. *J. Biol. Chem*. published ahead of print.

Mootha, V.K., Bunkenborg, J., Olsen, J.V., Hjerrild, M., Wisniewski, J.R., Stahl, E., Bolouri, M.S., Ray, H.N., Sihag, S., Kamal, M., Patterson, N., Lander, E.S. and Mann, M. (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell* **115** 629–640.

Müller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T.F. and Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. *EMBO J*. **18**, 339–352.

Müller, A., Gunther, D., Brinkmann V., Hurwitz R., Meyer T.F. and Rudel T. (2000). Targeting of the pro-apoptotic VDAC-like porin (PorB) of *Neisseria gonorrhoeae* to mitochondria of infected cells. *EMBO J* **19**, 5332–5343.

Müller, A., Rassow, J., Grimm, J., Machuy N., Meyer T.F., and Rudel T. (2002). VDAC and the bacterial porin PorB of *Neisseria gonorrhoeae* share mitochondrial import pathways. *EMBO J*. **21**, 1916–1929.

Nagai, T., Abe, A. and Sasakawa, C. (2004). Targeting of enteropathogenic *Escherichia coli* EspF to host mitochondria is essential for the bacterial pathogenesis: critical role of the 16$^{th}$ leucine residue in EspF. *J. Biol. Chem*. Published ahead of print.

Nakayama, M., Kimura, M., Wada, A., Yahiro, K., Ogushi, K., Niidome, T., Fujikawa, A., Shirasaka, A., Aoyama, N., Kurazano, H., Noda, M., Moss, J. and Hirayama, T. (2003). *Helicobacter pylori* VacA activates the p38/activating transcription factor 2-mediated signal pathway in AZ-521 cells. *J. Biol. Chem*. **279**, 7024–7028.

Nataro, J.P. and Kaper J.B. (1998). Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev*. **11**, 142–201.

Newmeyer, D.D. and Ferguson-Miller, S. (2003). Releasing power for life and unleashing the machineries of death. *Cell* **112**, 481–490.

Nougayrede, J.P. and Donnenberg, M.S. (2004). Enteropathogenic *Escherichia coli* EspF is targeted to mitochondria and is required to initiate the mitochondrial death pathway. *Cell Microbiol*. **6**, 1097–1111.

Papini, E., Zoratti, M. and Cover, T.L. (2001). In search of the *Helicobacter pylori* VacA mechanism. *Toxicon*, **39**, 1757–1767.

Paschen, S.A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D. and Neupert, W. (2003). Evolutionary conservation of biogenesis of beta-barrel membrane proteins. *Nature* **426**, 862–866.

Patel, H.K., Willhite, D.C., Patel, R.M., Ye, D., Williams, C.L., Torres, E.M., Marty, K.B., MacDonald, R.A. and Blanke, S. (2002). Plasma cholesterol modulates cellular vacuolation induced by *Helicobacter pylori* vacuolating cytotoxin. *Infect. Immun*. **70**, 4120–4123.

Petit, P., Breard, J., Montalescot, V., El Hadj, N.B., Levade, T., Popoff, M.R. and Geny, B. (2003). Lethal toxin of *Clostridium sordellii* induces apoptotic cell death by disruption of mitochondrial homeostasis in HL-60 cells. *Cell Microbiol*. **5**, 761–771.

Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E. and Boquet, P. (2000). High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored proteins and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol. Biol. Cell* **11**, 3897–3909.

Ricci, J.E., Munoz-Pinedo, C. and Fitzgerald, P. (2004). Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell* **117**, 773–786.

Rizzuto, R., Pinton, P., Carrington, W., Fay, F.S., Fogarty, K.E., Lifshitz, L.M., Tuft, R.A. and Pozzan, T. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. *Science* **280**, 1763–1766.

Rizzuto, R., Duchen, M.R. and Pozzan, T. (2004). Flirting in little space: the ER/mitochondria Ca2+ liaison. *Sci. STKE* **215**, 1.

Rudel, T., Schmid, A., Benz, R., Kolb, H.A., Lang, F. and Meyer, T.F. (1996). Modulation of Neisseria porin (PorB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. *Cell* **85**, 391–402.

Schraw, W., Li, Y., McClain, M.S., van der Goot, F.G. and Cover, T.L. (2002). Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. *J. Biol. Chem*. **277**, 34642–34650.

Shirin, H. and Moss, S.F (1998). *Helicobacter pylori* induced apoptosis. *Gut* **43**, 592–594.

Sonawane, N.D., Szoka, F.C. Jr. and Verkman, A.S. (2003). Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem*. **278**, 44826–44831.

Vinion-Dubiel, A.D., McClain, M.S., Czajkowsky, D.M., Iwamoto, H., Ye, D., Cao, P., Schraw, W., Szabo, G., Blanke, S.R., Shao, Z. and Cover T.L. (1999). A dominant negative mutant of *Helicobacter pylori* vacuolating toxin (VacA) inhibits VacA-induced cell vacuolation. *J. Biol. Chem*. **274**, 37736–37742.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727–730.

Weel, J.F. and van Putten, J.P. (1991). Fate of the outer membrane protein P.IA in early and late events of gonococcal infection of epithelial cells. *Res. Microbiol*. **142**, 985–993.

Wiedemann, N., Frazier, A.E. and Pfanner, N. (2004). The protein import machinery of mitochondria. *J. Biol. Chem*. **279**, 14473–1446.

Willhite, D.C., Cover, T.L. and Blanke, S.R. (2003). Cellular vacuolation and mitochondrial cytochrome c release are independent outcomes of *Helicobacter pylori* vacuolating cytotoxin activity that are each dependent on membrane channel formation. *J. Biol. Chem*. **278**, 48204–48209.

Willhite, D.C., and Blanke, S.R. (2004). *Helicobacter pylori* vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. *Cell Microbiol*. **6**, 143–154.

Yaffe, M.P. (1999). The machinery of mitochondrial inheritance and behavior. *Science* **283**, 1493–1497.

Yahiro, K. Wada, A., Nakayama, M., Kimura, T., Ogushi, K., Niidome, T., Aoyagi, H., Yoshino, K., Yonsawa, K., Moss, J. and Hiramaya, T. (2003). Protein tyrosine phosphatese α, RPTPα, is a *Helicobacter pylori* VacA receptor. *J. Biol. Chem*. **278**, 19183–19189.

Yi, M., Weaver, D. and Hajnoczky, G. (2004). Control of mitochondrial motility and distribution by calcium signal: a homeostatic circuit. *J. Cell Biol*. **167**, 661–672.

Zhang, A.S., Sheftel, A.D. and Ponka, P. (2004). Intracellular kinetics of iron in reticulocytes: evidence for endosome involvement in iron targeting to mitochondria. *Blood*, published ahead of print.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R. and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev*. **15**, 1481–1486.

CHAPTER

# 11

# Toxins activating Rho GTPases and exploiting the cellular ubiquitin/proteasome machineries

*Michel Gauthier, Gilles Flatau, Patrice Boquet, and Emmanuel Lemichez*

## INTRODUCTION

One of the most fascinating aspects of bacterial factors modifying Rho GTPases follows from the findings that, in addition to Rho inactivating factors, we have identified a growing family of activating factors. These factors are either toxins or factors directly injected into cells by cell-bound bacteria. The diversity and multiplicity of bacterial factors activating Rho proteins clearly highlight their importance for bacteria in establishing a tight relationship with their host. Toxins activating Rho proteins are closely related to each other. They share a high level of sequence homology and molecular mechanism of action. These bacterial factors were termed "toxins" to refer to their mechanism of penetration into cells. Progress made on the knowledge of their mechanism of action now raises questions on the nature of these bacterial factors, which likely represent bacterial factors for persistence in the host. Supporting this notion are the recent discoveries concerning the cellular mechanism of action of the CNF1 toxin. It catalyzes the permanent activation of Rho GTPases by post-translational modification. In cells, it has been recently established that Rho proteins, upon activation by CNF1, are rapidly ubiquitinylated and conveyed to the proteasomal machinery for proteolysis. This counterintuitive mechanism of action results in a moderate activation of Rho proteins. Host cells respond to the activation of Rho proteins by producing a large array of immunomodulators. Based on these findings it is likely that, while Rho protein activation allows bacterial internalization into cells, a decrease in the level of Rho protein activation lowers the host inflammatory responses.

## THE FAMILY OF RHO GTPASE–ACTIVATING TOXINS

To date, bacterial toxins activating Rho proteins have been isolated exclusively from human and animal pathogenic Gram-negative bacteria. These bacterial products were first purified based on their dermonecrotic (or necrotizing) activity when injected subcutaneously and at high doses in guinea pigs, mice, or rabbits (Caprioli *et al*., 1983; Horiguchi *et al*., 1989). Cloning and sequence analysis of these factors revealed their organization similarity to other known toxins (Falbo *et al*., 1993; Horiguchi, 2001). This toxin family encompasses different toxins found in either species belonging to *Bordetella* (dermonecrotic toxins, DNTs) or *Escherichia* and *Yersinia* species (cytotoxic necrotizing factors, CNF1, CNF2, or CNFy).

CNF1 is a chromosomally encoded protein of 1,014 amino acids with a predicted molecular mass 113.7 kDa. It is found in 30% of uropathogenic *E. coli* isolates (Landraud *et al*., 2000). CNF2, isolated from calves' and piglets' pathogenic *E. coli*, is a 110 kDa protein that shares about 90% identity with CNF1 (De Rycke *et al*.,

1999). More recently, Lockman and coworkers have described a CNF-related toxin in *Yersinia pseudotuberculosis,* namely CNFy, which bears 65.1% sequence identity with CNF1 (Lockman *et al*., 2002). Finally, the dermonecrotic toxins (DNTs) have been isolated from *Bordetella pertussis, B. parapertussis,* and *B. bronchiseptica* (Horiguchi, 2001). The DNT genes of *B. pertussis* and *B. bronchiseptica* were found to be more than 99% identical (Walker and Weiss, 1994; Pullinger *et al*., 1996). DNT of *B. bronchiseptica* is a protein of 1,464 amino acids, which shares sequence homologies with CNFs restricted to its catalytic domain (Lemichez *et al*., 1997; Horiguchi, 2001).

The *cnf1* gene is located within the pathogenicity island (PAI) PAI-II of the J96 UPEC strain, where it lies between the *hly* and *prs* operons (Swenson *et al*., 1996). At the difference with *cnf1* and *cnfy*, which are chromosomally encoded, *cnf*2 is located on a plasmid (Oswald and De Rycke, 1990; De Rycke *et al*., 1999). The genetic link between genes encoding CNF1 and α-hemolysin toxin reflects a co-regulation in their transcription (Landraud *et al*., 2003). In the *E. coli* J96 strain, transcription of *cnf1* is initiated from the *hly* promoter and requires the RfaH antiterminator factor activity (Landraud *et al*., 2003). RfaH binds to a specific nucleotide sequence found in the *hly* promoter (*ops/JUMP start*), where it interacts with the RNA polymerase and confers to the enzyme an antiterminator activity (Santangelo and Roberts, 2002). It is established that the α-hemolysin operon *hlyCABD* is transcribed in a single mRNA under the positive control of RfaH (Leeds and Welch, 1996). The transcription of the *cnf1* gene likely results from the formation of a polycistronic *hlyCABDcnf1* mRNA (Landraud *et al*., 2003).

## CNF1 AND DNT STRUCTURE/FUNCTION RELATIONSHIP

### Organization of CNF1 and DNT toxins in functional domains

CNF1 and DNT toxins are synthesized as unique polypeptides, which are organized in three functional domains (Figure 11.1) (Lemichez *et al*., 1997; Kashimoto *et al*., 1999). The amino-terminal parts of both toxins contain the cell receptor-binding domain (Lemichez *et al*., 1997; Kashimoto *et al*., 1999). The cell-binding domain of CNF1 has been delineated to the first 190 amino-terminal residues, whereas for DNT it is comprised in the first 53 amino-terminal residues (Fabbri *et al*., 1999; Matsuzawa *et al*., 2004). CNF1 binds tightly to its receptor, with a Kd as low as $2.10^{-11}$ M on HEp-2 cells (Contamin *et al*., 2000). The receptor-binding domain of CNF1 is followed by a domain containing two hydrophobic helices. Based on homologies to diphtheria toxin (DT), it has been hypothesized that this part of CNF1 might correspond to a membrane-anchoring domain, allowing the initiation of the translocation of catalytic domain into the cell cytosol (Figure 11.1) (Lemichez *et al*., 1997). In the case of DT, negatively-charged residues located between the two hydrophobic helices TH8–9 are protonated at acidic pH (O'Keefe *et al*., 1992). This allows the insertion of TH8–9 into the lipid bilayer (Wang *et al*., 1997). This event likely initiates the translocation of the catalytic domain. Mutagenesis analysis of the inter-region of the CNF1 hydrophobic helices has confirmed the analogy between both toxins (Pei *et al*., 2001). Finally, the most carboxy-terminal part of CNF1 and DNT contains the enzymatic domain. The catalytic domain of CNF1 has been crystallized (Buetow *et al*., 2001). The CNF1 deamidase domain form a narrow (15×30 $Å^2$) and deep pocket of about 10 Å, in which is located the catalytic triad corresponding to residues V833, C866, and H881. Consistently, mutations C866S and H881A abolished the toxin enzymatic activity (Schmidt *et al*., 1998). The tight structure of this pocket likely ensures a restriction of the accessibility of the glutamine 63 of the switch-II region (residues 59–78) of RhoA and thus confers the specificity of modification of Rho proteins by CNF1 (Flatau *et al*., 2000; Buetow *et al*., 2001). Other determinants, such as the flexible loops lining the entrance of the pocket of catalytic domain of CNF1, may confer the specificity of Rho protein recognition. Consistently, it has been observed that deletion of loop-8 (residues 964–970) and loop-9 (residues 996–1003) dramatically abolished the deamidation activity of the catalytic domain of CNF1, whereas this activity showed a significant increase upon deletion of loop-2 (residues 790–795). These results suggest that whereas loops -8 and -9 might be directly involved in RhoA recognition and/or binding, loop-2 might have to rearrange during RhoA binding (Buetow and Ghosh, 2003). Modelization of the catalytic domain of DNT suggests that, in contrast to that of CNF1, the pocket contains a negative charge that may attract positively charged primary amines (Buetow *et al*., 2001). This difference likely accounts for the transglutaminase activity of DNT (Buetow *et al*., 2001).

### CNF1 and DNT translocation into the cell cytosol

Despite their close organization, CNF1 and DNT toxins have evolved two different mechanisms to inject their catalytic domain into cells (Figure 11.2). CNF1 binds to a cell surface receptor, which was identified as

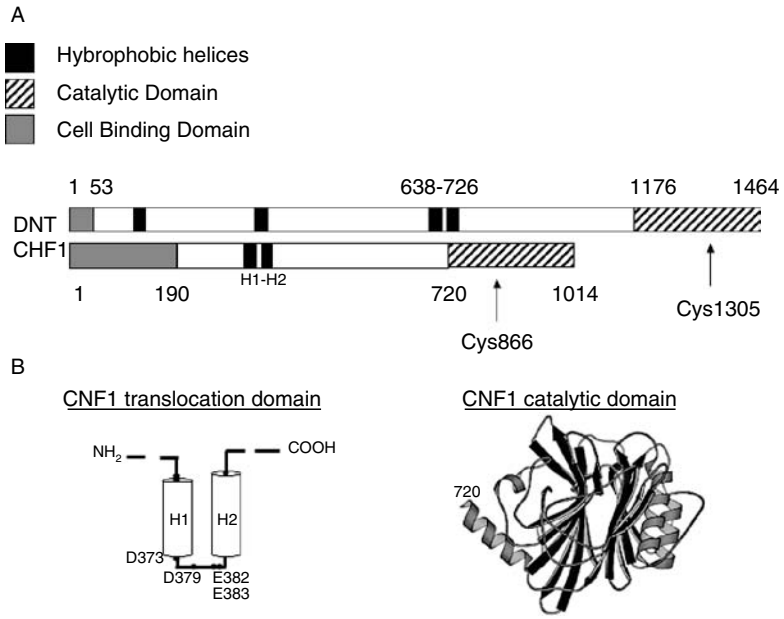



**FIGURE 11.1** Organization of CNF1 and DNT toxins in functional domains. A. Schematic representation of the organization of CNF1 and DNT toxins. B. CNF1 translocation domain: this shows a representation of the two helices of CNF1, which probably act by anchoring the toxin into the lipid bilayer at acidic pH to initiate the translocation of the catalytic domain into the cell cytosol (Pei *et al.*, 2001). CNF1 catalytic domain: this shows a representation of the structure of the catalytic domain of CNF1 (residues 720-1014) (Buetow *et al.*, 2001).

the 67 kDa laminin receptor (Chung *et al.*, 2003; Kim *et al.*, 2004). It remains to be determined whether this cellular receptor actually represents the high affinity receptor to CNF1. In contrast to CNF1, no DNT receptor has been isolated to date. The binding of both toxins to a cell surface receptor allows their internalization into endocytic vesicles at low rate. CNF1 enters endocytic vesicles by a non-clathrin endocytosis (independent of Eps15, dynamin, or intersectin-Src homology 3) (Contamin *et al.*, 2000). Following its entry into endocytic vesicles, CNF1 reaches late endosomal compartments. The acidic conditions found in late endosomes allow the transfer of the catalytic domain to the cytosol (Contamin *et al.*, 2000; Pei *et al.*, 2001). It remains to be determined which part of the molecule reaches the cytosol. At the difference with other known bacterial toxins, DNT intoxicates cells by an original mechanism, independent of endosome acidification (bafilomycin A1 insensitive) and of its transfer to the endoplasmic reticulum (brefeldin A insensitive) (Matsuzawa *et al.*, 2004). DNT enters endocytic compartments by a dynamin-dependent endocytosis, where the amino-terminal domain (residues 1–53) is likely cleaved by furin. It is thought that cleavage of the last 53 amino acid residues might unmask hydrophobic helices of the toxin and thus initiate the translocation of the catalytic domain into the cytosol (Matsuzawa *et al.*, 2002).

## CNF1 and DNT-catalyzed deamidation/transglutamination of Rho proteins

Following the initial discovery that CNF1 intoxication of cells resulted in a massive reorganization of the actin cytoskeleton (Fiorentini *et al.*, 1988) and that both CNF1 and DNT induced a mobility shift of RhoA on SDS-PAGE (Fiorentini *et al.*, 1994; Oswald *et al.*, 1994; Horiguchi *et al.*, 1995) , it was hypothesized that these toxins might catalyze a direct post-translational modification of Rho (Fiorentini *et al.*, 1995; Horiguchi *et al.*, 1995). Soon after, it was discovered through different approaches that CNF1 catalyzes the deamidation of the glutamine 63 of RhoA into a glutamic acid (Flatau *et al.*, 1997; Schmidt *et al.*, 1997). At the difference with CNF1, the DNT toxin preferentially produced the transglutamination of RhoA (Figure 11.3) (Schmidt *et al.*, 1998; Masuda *et al.*, 2000). Both biochemical reactions are similar except that deamidation uses $H_20$ as acceptor molecules instead of amine molecules used for transglutamination. The Rho sequence determinants responsible for CNF1 recognition and modification were identified by constructing chimeric molecules between RhoA and Ha-Ras, a more distant small GTPase not modified by CNF1. The specificity of deamidation is conferred by the residues R68 and L72 of RhoA (Lerm *et al.*, 1999a). These residues are found at this position specifically in members of the Rho

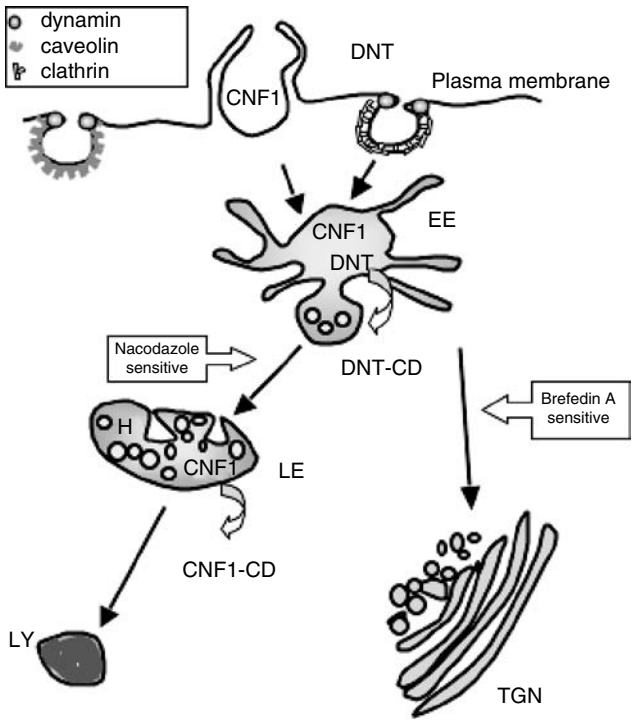



**FIGURE 11.2** CNF1 and DNT penetration into host cells. CNF1 and DNT cell intoxication depicting the endosomal compartments EE (early endosome), LE (late endosome), LY (lysosome), and TGN (trans-Golgi network). CNF1 binds to a cell surface receptor. This carrier protein allows the toxin to reach endosomal compartments by a pathway, which is blocked neither with filipin (caveolae-like) nor by dominant negative mutants of Eps15, dynamin, or intersectin-Src homology 3 (clathrin-coated-vesicles). Cell internalization of the toxin is followed by its reaching to a late endosomal compartment. The acidic conditions of the late endosome allow the transfer of the catalytic domain of the toxin into the cytosol (noted CNF1-CD). Intoxication of cells by DNT, at the difference with CNF1, does not require endosomal acidic conditions (Bafilomycin A1 insensitive). The transfer of the catalytic domain (DNT-CD) likely occurs in an early endosomal compartment, which reaching is independent from actin cytoskeleton (Cytochalasin D insensitive), the microtubules (Nocodazole insensitive), or a retrograde transfer to the reticulum (Brefeldin A insensitive).

subfamily such as RhoA, Rac1, and Cdc42 (Lerm *et al*., 1999a). Consequently, Rac1 and Cdc42 are also deamidated by CNF1 *in vivo* (Lerm *et al*., 1999b).

The glutamine-63 of RhoA modified by CNF1 is a critical amino-acid conserved in all known proteins of the Ras superfamily (Takai *et al*., 2001). This glutamine residue had long been identified as a hot spot of oncogenic mutations in HRas. Its mutation is responsible for impairing the HRas GTPase activity (Der *et al*., 1986). Similarly, the CNF1-catalyzed deamidation of the glutamine 63 of RhoA was indeed found to block its GTPase activity, thereby conferring Rho permanent activation (Flatau *et al*., 1997; Schmidt *et al*., 1997).

## *In vivo* molecular activity of CNF1 and DNT

Studying the extent of Rho protein activation in a model of bladder epithelial cells in order to determine the specificity of Rho activation by CNF1, it was observed that cell intoxication resulted in a transient (instead of permanent) activation of Rho proteins (Doye *et al*., 2002). In this cellular model, CNF1 produced a maximal activation of Rac and Cdc42 isoforms (Doye *et al*., 2002). The transient activation of Rac was directly correlated to the proteasomal degradation of the form activated by CNF1 (Figure 11.2) (Doye *et al*., 2002; Lerm *et al*., 2002). Prior to degradation by the proteasome, cellular proteins are epitope-tagged by conjugation with a polyubiquitin string (Finley *et al*., 2004). Proteasomal degradation of endogenous Rac was found to follow the classical formation of a K48-polyubiquitin chain (Doye *et al*., 2002). Rac sensitivity to ubiquitin-mediated proteasomal degradation correlated with the strength of its activation (Doye *et al*., 2002; Boyer and Lemichez, 2004). In addition it was shown that Rac activation by the GEF domain of Dbl also resulted in a significant increase of Rac ubiquitinylation sensitivity (Doye *et al*., 2002). These results suggested that ubiquitinylation of Rho proteins might correspond to a yet unraveled regulation of these proteins that CNF1 may have hijacked. This idea is now sustained by the finding that the Smad ubiquitinylation-related factor 1 (Smurf1) bears an ubiquitin ligase activity on RhoA (Wang *et al*., 2003). It has been hypothesized that Rho ubiquitinylation might occur during mislocalized activation of RhoA, especially in membrane ruffles where activated RhoA might have antagonized the activity of Rac (Wang *et al*., 2003).

Recent findings suggest a more complex relationship between Rho protein activation and their ubiquitin-mediated proteasomal degradation. For instance, it has been reported that DNT activates but does not produced the proteasomal degradation of Rac in HeLa cells (Pop *et al*., 2004). These discrepancies probably reflect differences in the efficiencies of cell intoxication by CNF1 and DNT toxins. In fact, the proteasomal degradation of Rac can be detected when the kinetic of deamidation/degradation is higher than its kinetic of synthesis. For instance, a 24-hour cell intoxication by CNF1 produces a cellular depletion of Rac clearly visible at concentrations higher than $10^{-10}$ M (Munro *et al*., 2004). In addition, it has also been reported that CNFy specifically activates RhoA in HeLa cells, whereas it impairs the GTPase activity of RhoA, Rac1, and Cdc42 *in vitro* (Hoffmann *et al*., 2004). These reports, together with other findings showing that Rac is activated without being degraded in HEp-2 cells, raise open questions (Doye *et al*., 2002). For instance, these differences

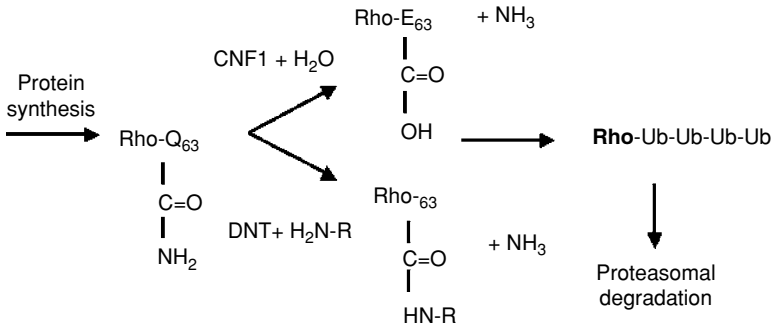



**FIGURE 11.3** Enzymatic activity of Rho GTPase, activating toxins. CNF1, 2, and CNFy have a deamidase activity responsible for the post-translational mutation of the glutamine 63 of RhoA and its equivalent 61 in Rac and Cdc42 into a glutamic acid. This mutation impairs the intrinsic GTPase activity of Rho proteins, resulting in their permanent activation. The activation of Rho proteins sensitizes them to the ubiquitin-mediated proteasomal degradation (Doye *et al.*, 2002). Similarly, DNT catalyzes the transglutamination of the glutamine 63 of RhoA (61 in Rac and Cdc42). The effects of CNF1 on Rho proteins result from different kinetics and are a direct consequence of the toxin concentration. If the reaction of deamidation and ubiquitin-mediated proteasomal degradation is higher to that of Rho protein synthesis, it produces a robust and transient activation of Rho proteins resulting from the cellular depletion of Rho proteins. This effect occurs at high concentrations of CNF1. In contrast, at lower concentrations of CNF1, the deamidation and ubiquitin-mediated proteasomal degradation of Rho proteins allow their moderate activation without visible cellular depletion.

might be attributed to the cell type specificity. One possibility could be that CNF1 specifically triggers Rho, Rac, and Cdc42 ubiquitin-mediated degradation in epithelial bladder and endothelial cells (Doye *et al.*, 2002; Munro *et al.*, 2004). These observations may account for the findings that CNF1 is preferentially encountered in *E. coli* strains responsible for urinary or meningitis infections. A more trivial explanation would be that some cancer cell lines might have a lower activity of ubiquitinylation of one (or more) Rho protein isoform(s). This would raise important questions concerning a possible relationship between ubiquitinylation of Rho proteins and cancer.

## CELLULAR EFFECTS OF RHO ACTIVATION/DEGRADATION BY BACTERIAL TOXINS

### Biological activities of Rho proteins

Rho proteins have turned out to be a major subject of research for both microbiologists and cell biologists since their first description as targets of *C. botulinum* C3 exoenzyme and as master transducers of the regulation of the actin cytoskeleton by growth factors (Chardin *et al.*, 1989; Ridley and Hall, 1992; Ridley *et al.*, 1992; Ridley and Hall, 2004). To date, we know at least 20 bacterial virulence factors and toxins modifying Rho proteins (Boquet and Lemichez, 2003). Rho proteins belong to the Ras superfamily of small GTPases (Takai *et al.*, 2001; Burridge and Wennerberg, 2004). Upon GTP binding, Rho protein members associate with and activate specific effectors (Burridge and Wennerberg, 2004). These interaction specificities allow, for instance, Cdc42 and Rac1 to regulate actin filament assembly producing membrane filopodia or ruffles, respectively (Burridge and Wennerberg, 2004). In contrast, RhoA and RhoC control both the bundling of actin filaments and their contraction through the regulation of the activity of myosin (Burridge and Wennerberg, 2004). Studies made on the cross-talk between bacterial virulence and Rho proteins have highlighted the importance of these cellular proteins in controlling and organizing the cellular defenses against pathogenic bacteria (Boquet and Lemichez, 2003). Through their regulatory properties of actin filament polymerization, organization, and contractility, Rho proteins control a large array of cell processes requiring cell shaping and membrane dynamics (Etienne-Manneville and Hall, 2002). For instance, Rho proteins control the cohesion of cells either between each other or at the contact with the cellular matrix (Etienne-Manneville and Hall, 2002). This aspect is of importance for pathogenic bacteria, of which penetration into host cells is frequently limited due to the basolateral localization of their cell-internalization receptors (Cossart and Sansonetti, 2004). Rho proteins also participate in the control of cell cycle progression and apoptosis (Sahai and Marshall, 2002). This last aspect is also closely related to pathogen requirements. For instance, it is thought that apoptosis inhibition in target cells may favor bacterial persistence at the epithelium surface, whereas apotosis

may favor bacterial spreading inside tissues (Mulvey *et al*., 1998). Rho proteins also regulate cell motility and differentiation (Etienne-Manneville and Hall, 2002). These aspects are of particular interest in relationship to cellular effectors of the immune response. For instance, macrophage chemotactism up to the site of bacterial infection is under the control of the actin cytoskeleton machinery, as is the phagocytosis bacteria by macrophages (Caron and Hall, 1998).

## Biological activities of CNF1 and DNT toxins

Whether DNTs and CNFs play similar roles in the bacterial pathology, with respect to *Bordetella* and *Escherichia* productive strains, remains to be investigated. DNT studies in animal models have clearly highlighted the importance of this toxin in turbinate atrophy and pneumonia pathology (Magyar *et al*., 2000; Brockmeier *et al*., 2002), whereas studies carried on CNF1 have rather investigated its effects on cellular models. Major progress has been made in our knowledge of molecular mechanism of invasive factors of Gram-negative bacteria (Cossart and Sansonetti, 2004). Importantly, all these bacterial cell-invasion strategies have in common the activation of a family of cellular regulators expressed in all cells, namely the Rho GTPases. In that respect, the CNF1 toxin represents an interesting model. CNF1 produces a counterintuitive mechanism consisting of Rho protein activation responsible for sensitizing them to ubiquitin-mediated proteasomal degradation (Figure 11.2) (Doye *et al*., 2002). These observations raise the question of the importance of both Rho protein activation and degradation in bacterial virulence. It has been shown that activation of Rho proteins is necessary to induce the CNF1-triggered internalization of bacteria by epithelial and endothelial cells (Falzano *et al*., 1993; Doye *et al*., 2002; Kim *et al*., 2004). Interestingly, the reaching of a low level of Rho protein activation due to equilibrium between activation and degradation was shown to confer higher invasive properties to pathogenic bacteria (Figure 11.4). Similar requirements were also found to confer intercellular junction dismantling and epithelial cell motility inside monolayers (Doye *et al*., 2002). Urinary tract infections (UTIs) have long been considered as acute and often self-limiting infections caused by non-invasive *E. coli*. Nevertheless, growing evidence suggests that UPECs are individuals capable of colonizing the bladder mucosa by a process requiring the coordinate action of different virulence factors (Anderson *et al*., 2004). At first, bacteria attach to bladder epithelial cells through adhesins, preventing them from miction clearing. Persistence of bacteria probably also requires to escape host defenses, comprising innate effectors such as the membrane attack complex of the complement system and immune-cell effectors. New evidence suggests that protection of UPECs against host defenses may be achieved by limiting the impact of host innate responses, for instance, by invading cells (Schilling *et al*., 2001). In fact, intracellular invasion of epithelial cells may not only protect UPECs against host defenses but may also allow bacteria to replicate and/or persist into host cells (Mulvey *et al*., 1998; Svanborg *et al*., 1999). Further studies will have to clarify whether CNF1 is a major determinant of recurrent cystitis, which might then be due to formation of UPECs reservoir in epithelial cells during infection.

Studying the cell response to intoxication by CNF1 revealed that intoxication interferes with classical signaling pathways, such as the c-Jun N-terminal kinase and NF-κB pathways, leading to gene regulation (Lerm *et al*., 1999b; Boyer *et al*., 2004; Munro *et al*., 2004). Consequently, high levels of cell intoxication by CNF1 trigger a gene response consisting of a selective activation of about 0.19% of the 33,000 genes probed on DNA arrays (Munro *et al*., 2004). The 10 most CNF1-activated genes formed a coherent family of inflammatory mediators aiming at leukocyte recruitment and activation (Figure 11.4). These results are in agreement with a possible effect of CNF1 in the development of UPECs infection, which results in an acute inflammatory disease. Induction by CNF1 of membrane metalloproteases and syndecan family products may contribute to the recruitment of leukocytes to the site of infection. Other identified inflammatory regulators, such as PLAU-urokinase (for lumen arteries restriction), as well as Prostaglandin G/H synthesis enzymes, may complete the task of leukocytes recruitment and activation to the site of bacterial infection. Production of GRO-family and MIP-3α chemokines may participate in recruiting lymphocytes and antigen presenting dendritic cells, respectively. CNF1-intoxicated cells also produce innate-defense effectors, comprising complement factor-3 for pathogen phagocytosis, and factor-9 (a component of the cytolytic membrane attack complex), as well as GliPR, a plant pathogen related-1 protein homologue. Finally, the transcriptome of CNF1-intoxicated HUVECs appears to share many similarities with known TNFα-regulated genes, among them TRAF1, cIAP2, and A20.

Interestingly, the transcriptome analysis of cells intoxicated by CNF1 points to Rac/Cdc42 activation being responsible for the production of inflammatory mediators (Munro *et al*., 2004). For instance IL8, MCP-1, and MIP-3α were found to be were expressed as a function of the levels of activated-Cdc42 or Rac (Munro *et al*., 2004). These findings are consistent with other studies showing that Rac and Cdc42 are engaged in Toll-like receptors (TLRs) signal transduction (Arbibe

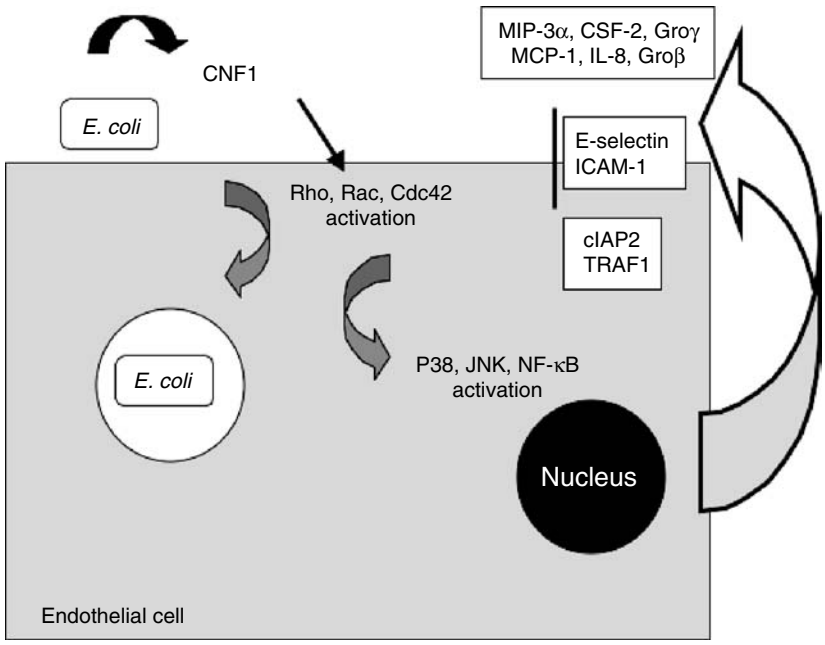



**FIGURE 11.4** Relationship between *E. coli* producing CNF1 and the host cell genetic response. The intoxication of human umbilical vein endothelial cells HUVECs by CNF1 results in the activation of p38 and c-Jun kinases, as well as I-κB degradation for NF-κB activation. While CNF1 allows the internalization of *E. coli* into HUVECs, the activation of these signaling pathways engages a genetic program aimed at leukocytes' recruitment and activation. Here are depicted the 10 most CNF1-activated genes (Munro *et al*., 2004). Induction of inflammatory mediators for leukocytes' attraction and activation: MIP-3α, CSF-2, Groγ, MCP-1, IL-8, Groβ could be correlated to the production of the leukocyte cell-binding receptors E-Selectin and ICAM-1. Cell response to CNF1 also included TRAF1 (TNF-receptor associated protein-1) and cIAP2 (mammalian inhibitor of apoptosis protein-1 homologue C), two modulators of the TNF-α receptor signaling.

*et al*., 2000). Collectively, these results indicate that bacteria have evolved virulence systems aimed at producing a moderate activation of Rho proteins to delay and/or mit the cellular alarm program of the host, while invading cells. The moderate activation of Rho proteins, for bacterial internalization into cells, may thus be beneficial during early stages of the bacterial infection, prior to substantive bacterial growth and a resulting acute inflammatory reaction (Janssens and Beyaert, 2003; Anderson *et al*., 2004). During the acute phase of the inflammatory reaction, CNF1 may also play a role by acting directly on inflammatory cells or protecting cells from apoptosis (Fiorentini *et al*., 1997; Capo *et al*., 1998; Hofman *et al*., 2000; Malorni *et al*., 2003).

## ACKNOWLEDGMENTS

We are grateful to all members of our laboratory. We apologize to our colleagues whose excellent work could not be cited because of the lack of space. Our laboratory is funded by the French National Institute of Health and Medical Research (INSERM).

## REFERENCES

Anderson, G.G., Dodson, K.W., Hooton, T.M. and Hultgren, S.J. (2004). Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Trends Microbiol*. **12**, 424–430.

Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P.J., Ulevitch, R.J. and Knaus, U.G. (2000). Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat. Immunol*. **1**, 533–540.

Boquet, P. and Lemichez, E. (2003). Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? *Trends Cell Biol*. **13**, 238–246.

Boyer, L. and Lemichez, E. (2004). Targeting of host-cell ubiquitin and ubiquitin-like pathways by bacterial factors. *Nat. Rev. Microbiol*. **2**, 779–788.

Boyer, L., Travaglione, S., Falzano, L., Gauthier, N.C., Popoff, M.R., Lemichez, E., Fiorentini, C. and Fabbri, A. (2004). Rac GTPase instructs nuclear factor-kappaB activation by conveying the SCF complex and IkBalpha to the ruffling membranes. *Mol. Biol. Cell* **15**, 1124–1133.

Brockmeier, S.L., Register, K.B., Magyar, T., Lax, A.J., Pullinger, G.D. and Kunkle, R.A. (2002). Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory disease in swine. *Infect. Immun*. **70**, 481–490.

Buetow, L., Flatau, G., Chiu, K., Boquet, P. and Ghosh, P. (2001). Structure of the Rho-activating domain of *Escherichia coli* cytotoxic necrotizing factor 1. *Nat. Struct. Biol*. **8**, 584–588.

Buetow, L. and Ghosh, P. (2003). Structural elements required for deamidation of RhoA by cytotoxic necrotizing factor 1. *Biochemistry* **42**, 12784–12791.

Burridge, K. and Wennerberg, K. (2004). Rho and Rac take center stage. *Cell* **116**, 167–179.

Capo, C., Meconi, S., Sanguedolce, M.V., Bardin, N., Flatau, G., Boquet, P. and Mege, J.L. (1998). Effect of cytotoxic necrotizing factor-1 on actin cytoskeleton in human monocytes: role in the regulation of integrin-dependent phagocytosis. *J. Immunol.* **161**, 4301–4308.

Caprioli, A., Falbo, V., Roda, L.G., Ruggeri, F.M., and Zona, C. (1983). Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. *Infect. Immun.* **39**, 1300–1306.

Caron, E. and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282**, 1717–1721.

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989). The mammalian G protein rhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J.* **8**, 1087–1092.

Chung, J.W., Hong, S.J., Kim, K.J., Goti, D., Stins, M.F., Shin, S., Dawson, V.L., Dawson, T.M. and Kim, K.S. (2003). 37-kDa laminin receptor precursor modulates cytotoxic necrotizing factor 1-mediated RhoA activation and bacterial uptake. *J. Biol. Chem.* **278**, 16857–16862.

Contamin, S., Galmiche, A., Doye, A., Flatau, G., Benmerah, A. and Boquet, P. (2000). The p21 Rho-activating toxin cytotoxic necrotizing factor 1 is endocytosed by a clathrin-independent mechanism and enters the cytosol by an acidic-dependent membrane translocation step. *Mol. Biol. Cell.* **11**, 1775–1787.

Cossart, P. and Sansonetti, P.J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304**, 242–248.

De Rycke, J., Milon, A. and Oswald, E. (1999). Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. *Vet. Res.* **30**, 221–233.

Der, C.J., Finkel, T. and Cooper, G.M. (1986). Biological and biochemical properties of human rasH genes mutated at codon 61. *Cell* **44**, 167–176.

Doye, A., Mettouchi, A., Bossis, G., Clement, R., Buisson-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P. and Lemichez, E. (2002). CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho GTPase activation for bacterial host cell invasion. *Cell* **111**, 553–564.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629–635.

Fabbri, A., Gauthier, M. and Boquet, P. (1999). The 5′ region of *cnf1* harbors a translational regulatory mechanism for CNF1 synthesis and encodes the cell-binding domain of the toxin. *Mol. Microbiol.* **33**, 108–118.

Falbo, V., Pace, T., Picci, L., Pizzi, E. and Caprioli, A. (1993). Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. *Infect. Immun.* **61**, 4909–4914.

Falzano, L., Fiorentini, C., Donelli, G., Michel, E., Kocks, C., Cossart, P., Cabanie, L., Oswald, E. and Boquet, P. (1993). Induction of phagocytic behavior in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Mol. Microbiol.* **9**, 1247–1254.

Finley, D., Ciechanover, A. and Varshavsky, A. (2004). Ubiquitin as a central cellular regulator. *Cell* **116**, S29–32, 22 p. following S32.

Fiorentini, C., Arancia, G., Caprioli, A., Falbo, V., Ruggeri, F. M. and Donelli, G. (1988). Cytoskeletal changes induced in HEp-2 cells by the cytotoxic necrotizing factor of *Escherichia coli*. *Toxicon* **26**, 1047–1056.

Fiorentini, C., Giry, M., Donelli, G., Falzano, L., Aullo, P. and Boquet, P. (1994). *E. coli* cytotoxic necrotizing factor 1 increases actin assembly via the p21 Rho protein. *Zentralbl. Bakteriol. Suppl.* **24**, 404–405.

Fiorentini, C., Donelli, G., Matarrese, P., Fabbri, A., Paradisi, S. and Boquet, P. (1995). *Escherichia coli* cytotoxic necrotizing factor 1: evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. *Infect. Immun.* **63**, 3936–3944.

Fiorentini, C., Fabbri, A., Matarrese, P., Falzano, L., Boquet, P. and Malorni, W. (1997). Hinderance of apoptosis and phagocytic behavior induced by *Escherichia coli* cytotoxic necrotizing factor 1: two related activities in epithelial cells. *Biochem. Biophys. Res. Commun.* **241**, 341–346.

Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387**, 729–733.

Flatau, G., Landraud, L., Boquet, P., Bruzzone, M. and Munro, P. (2000). Deamidation of RhoA glutamine 63 by the *Escherichia coli* CNF1 toxin requires a short sequence of the GTPase switch 2 domain. *Biochem. Biophys. Res. Commun.* **267**, 588–592.

Hoffmann, C., Pop, M., Leemhuis, J., Schirmer, J., Aktories, K. and Schmidt, G. (2004). The *Yersinia pseudotuberculosis* cytotoxic necrotizing factor (CNFY) selectively activates RhoA. *J. Biol. Chem.* **279**, 16026–16032.

Hofman, P., Le Negrate, G., Mograbi, B., Hofman, V., Brest, P., Alliana-Schmid, A., Flatau, G., Boquet, P. and Rossi, B. (2000). *Escherichia coli* cytotoxic necrotizing factor-1 (CNF-1) increases the adherence to epithelia and the oxidative burst of human polymorphonuclear leukocytes but decreases bacteria phagocytosis. *J. Leukoc. Biol.* **68**, 522–528.

Horiguchi, Y. (2001). *Escherichia coli* cytotoxic necrotizing factors and *Bordetella* dermonecrotic toxin: the dermonecrosis-inducing toxins activating Rho small GTPases. *Toxicon* **39**, 1619–27.

Horiguchi, Y., Nakai, T. and Kume, K. (1989). Purification and characterization of *Bordetella bronchiseptica* dermonecrotic toxin. *Microb. Pathog.* **6**, 361–368.

Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. and Matsuda, M. (1995). *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. *J. Cell Sci.* **108 (Pt 10)**, 3243–3251.

Janssens, S. and Beyaert, R. (2003). Role of Toll-like receptors in pathogen recognition. *Clin. Microbiol. Rev.* **16**, 637–646.

Kashimoto, T., Katahira, J., Cornejo, W. R., Masuda, M., Fukuoh, A., Matsuzawa, T., Ohnishi, T. and Horiguchi, Y. (1999). Identification of functional domains of *Bordetella* dermonecrotizing toxin. *Infect. Immun.* **67**, 3727–3732.

Kim, K.J., Chung, J.W. and Kim, K.S. (2004) 67-kDa Laminin receptor promotes internalization of cytotoxic necrotizing factor 1-expressing *Escherichia coli* K1 into human brain microvascular endothelial cells. *J. Biol. Chem.* [Epub ahead of print].

Landraud, L., Gauthier, M., Fosse, T. and Boquet, P. (2000). Frequency of *Escherichia coli* strains producing the cytotoxic necrotizing factor (CNF1) in nosocomial urinary tract infections. *Lett. Appl. Microbiol.* **30**, 213–216.

Landraud, L., Gibert, M., Popoff, M.R., Boquet, P. and Gauthier, M. (2003). Expression of cnf1 by *Escherichia coli* J96 involves a large upstream DNA region including the hlyCABD operon, and is regulated by the RfaH protein. *Mol. Microbiol.* **47**, 1653–1667.

Leeds, J.A. and Welch, R.A. (1996). RfaH enhances elongation of *Escherichia coli* hlyCABD mRNA. *J. Bacteriol.* **178**, 1850–1857.

Lemichez, E., Flatau, G., Bruzzone, M., Boquet, P. and Gauthier, M. (1997). Molecular localization of the *Escherichia coli* cytotoxic necrotizing factor CNF1 cell-binding and catalytic domains. *Mol. Microbiol.* **24**, 1061–1070.

Lerm, M., Schmidt, G., Goehring, U.M., Schirmer, J. and Aktories, K. (1999a). Identification of the region of rho involved in substrate recognition by *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1). *J. Biol. Chem*. **274**, 28999–29004.

Lerm, M., Selzer, J., Hoffmeyer, A., Rapp, U.R., Aktories, K. and Schmidt, G. (1999b). Deamidation of Cdc42 and Rac by *Escherichia coli* cytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. *Infect. Immun*. **67**, 496–503.

Lerm, M., Pop, M., Fritz, G., Aktories, K. and Schmidt, G. (2002). Proteasomal degradation of cytotoxic necrotizing factor 1-activated rac. *Infect. Immun*. **70**, 4053–4058.

Lockman, H.A., Gillespie, R.A., Baker, B.D. and Shakhnovich, E. (2002). *Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor. *Infect. Immun*. **70**, 2708–2714.

Magyar, T., Glavits, R., Pullinger, G.D. and Lax, A.J. (2000). The pathological effect of the *Bordetella* dermonecrotic toxin in mice. *Acta Vet Hung*. **48**, 397–406.

Malorni, W., Quaranta, M.G., Straface, E., Falzano, L., Fabbri, A., Viora, M. and Fiorentini, C. (2003). The Rac-activating toxin cytotoxic necrotizing factor 1 oversees NK cell-mediated activity by regulating the actin/microtubule interplay. *J. Immunol*. **171**, 4195–4202.

Masuda, M., Betancourt, L., Matsuzawa, T., Kashimoto, T., Takao, T., Shimonishi, Y. and Horiguchi, Y. (2000). Activation of rho through a cross-link with polyamines catalyzed by *Bordetella* dermonecrotizing toxin. *Embo. J*. **19**, 521–530.

Matsuzawa, T., Kashimoto, T., Katahira, J. and Horiguchi, Y. (2002). Identification of a receptor-binding domain of *Bordetella* dermonecrotic toxin. *Infect. Immun*. **70**, 3427–3432.

Matsuzawa, T., Fukui, A., Kashimoto, T., Nagao, K., Oka, K., Miyake, M. and Horiguchi, Y. (2004). *Bordetella* dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. *J. Biol. Chem*. **279**, 2866–2872.

Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W. C., Heuser, J. and Hultgren, S. J. (1998). Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli. Science* **282**, 1494–1497.

Munro, P., Flatau, G., Doye, A., Boyer, L., Oregioni, O., Mege, J.L., Landraud, L. and Lemichez, E. (2004). Activation and proteasomal degradation of rho GTPases by cytotoxic necrotizing factor-1 elicit a controlled inflammatory response. *J. Biol. Chem*. **279**, 35849–35857.

O'Keefe, D.O., Cabiaux, V., Choe, S., Eisenberg, D. and Collier, R.J. (1992). pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349. Lys. *Proc. Natl. Acad. Sci. USA* **89**, 6202–6206.

Oswald, E. and De Rycke, J. (1990). A single protein of 110 kDa is associated with the multinucleating and necrotizing activity coded by the Vir plasmid of *Escherichia coli. FEMS Microbiol. Lett*. **56**, 279–284.

Oswald, E., Sugai, M., Labigne, A., Wu, H.C., Fiorentini, C., Boquet, P. and O'Brien, A. D. (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl. Acad. Sci. USA* **91**, 3814–3818.

Pei, S., Doye, A. and Boquet, P. (2001). Mutation of specific acidic residues of the CNF1 T domain into lysine alters cell membrane translocation of the toxin. *Mol. Microbiol*. **41**, 1237–1247.

Pop, M., Aktories, K. and Schmidt, G. (2004). Isotype-specific degradation of Rac activated by the cytotoxic necrotizing factor 1. *J. Biol. Chem*. **279**, 35840–35848.

Pullinger, G.D., Adams, T.E., Mullan, P.B., Garrod, T.I. and Lax, A.J. (1996). Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of Bordetella spp. *Infect. Immun*. **64**, 4163–4171.

Ridley, A.J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389–399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401–410.

Ridley, A.J. and Hall, A. (2004). Snails, Swiss, and serum: the solution for Rac 'n' Rho. *Cell* **116**, S23–25, 22 p. following S25.

Sahai, E. and Marshall, C.J. (2002). RHO-GTPases and cancer. *Nat. Rev. Cancer* **2**, 133–142.

Santangelo, T.J. and Roberts, J.W. (2002). RfaH, a bacterial transcription antiterminator. *Mol. Cell* **9**, 698–700.

Schilling, J.D., Mulvey, M.A. and Hultgren, S.J. (2001). Dynamic interactions between host and pathogen during acute urinary tract infections. *Urology* **57**, 56–61.

Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M. and Aktories, K. (1997). Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. *Nature* **387**, 725–729.

Schmidt, G., Selzer, J., Lerm, M. and Aktories, K. (1998). The Rho-deamidating cytotoxic necrotizing factor 1 from *Escherichia coli* possesses transglutaminase activity. Cysteine 866 and histidine 881 are essential for enzyme activity. *J. Biol. Chem*. **273**, 13669–13674.

Svanborg, C., Godaly, G. and Hedlund, M. (1999). Cytokine responses during mucosal infections: role in disease pathogenesis and host defence. *Curr. Opin. Microbiol*. **2**, 99–105.

Swenson, D.L., Bukanov, N.O., Berg, D.E. and Welch, R.A. (1996). Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infect. Immun*. **64**, 3736–3743.

Takai, Y., Sasaki, T. and Matozaki, T. (2001). Small GTP-binding proteins. *Physiol. Rev*. **81**, 153–208.

Walker, K.E. and Weiss, A.A. (1994). Characterization of the dermonecrotic toxin in members of the genus. *Infect. Immun*. **62**, 3817–3828.

Wang, H.R., Zhang, Y., Ozdamar, B., Ogunjimi, A.A., Alexandrova, E., Thomsen, G.H. and Wrana, J.L. (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. *Science* **302**, 1775–1779.

Wang, Y., Malenbaum, S.E., Kachel, K., Zhan, H., Collier, R.J. and London, E. (1997). Identification of shallow and deep membrane-penetrating forms of diphtheria toxin T domain that are regulated by protein concentration and bilayer width. *J. Biol. Chem*. **272**, 25091–25098.

SECTION II

# BACTERIAL PROTEIN TOXINS ACTING IN THE INTRACELLULAR COMPARTMENT OF EUKARYOTIC CELLS

CHAPTER

# 12

# Molecular, functional, and evolutionary aspects of ADP-ribosylating toxins

*Vega Masignani, Mariagrazia Pizza, and Rino Rappuoli*

## INTRODUCTION

The mono-ADP-ribosylation reaction is an enzymatic activity that was initially discovered and studied at the molecular level in bacterial toxins. For a while this reaction was believed to be a peculiarity of bacteria and no role was known for it on normal metabolism of eukaryotic cells. Recently, many enzymes with ADP-ribosylating activity have been discovered in eukaryotic cells. Examples are mammalian ART1-5 and the rodent RT6. This growing family of enzymes shows that ADP-ribosylation is also an enzymatic reaction with an important role in the posttranslational modification of the eukaryotic cells.

ADP-ribosylating toxins are a variety of bacterial proteins with totally unrelated structures that have in common only one feature: they contain an enzyme with ADP-ribosyltransferase activity. The toxins with this activity are shown in Figure 12.1. The enzymatic moiety, which is represented by the black "A" domain in Figure 12.1, is responsible for the toxic effect. This domain is contained in a protein of approximately 20–25 kDa, but in some cases the size can be greater. Based on their overall structure the toxins can be divided into A/B toxins, binary toxins, or A/only toxins. A/B and binary toxins are molecules released by bacteria in the extracellular environment. The A domain contains the enzymatic toxic activity, while the B domain is a non-toxic part that functions as a carrier or delivery system for the A domain: it binds the receptor on the surface of eukaryotic cells and helps its translocation within the cell.

As shown in Figure 12.1, the A/B toxins are the best studied, and we know the three-dimensional structure of most of them. In some cases, the B domain can be divided into the B (cell binding) and T (membrane translocation domain).

The binary toxins have a similar organization; however, in this case the A and B domains are separately secreted on the culture supernatant. The B domain binds to the receptor on the surface of the target of the eukaryotic cells and only then is able to bind the A domain and help its translocation into the cytosol. Toxins with this structure are the C2 toxin of *Clostridium botulinum* and the related toxins shown in Figure 12.1.

The A/only toxins are those toxins constituted uniquely by the A domain with the enzymatic activity. A typical example of this type of toxin is the Exoenzyme S of *Pseudomonas aeruginosa* that is directly injected by bacteria into eukaryotic cells by a specialized secretion system. Other toxins with this structure have a still unknown mechanism of cell entry (Exoenzyme C3 of *C. botulinum*).

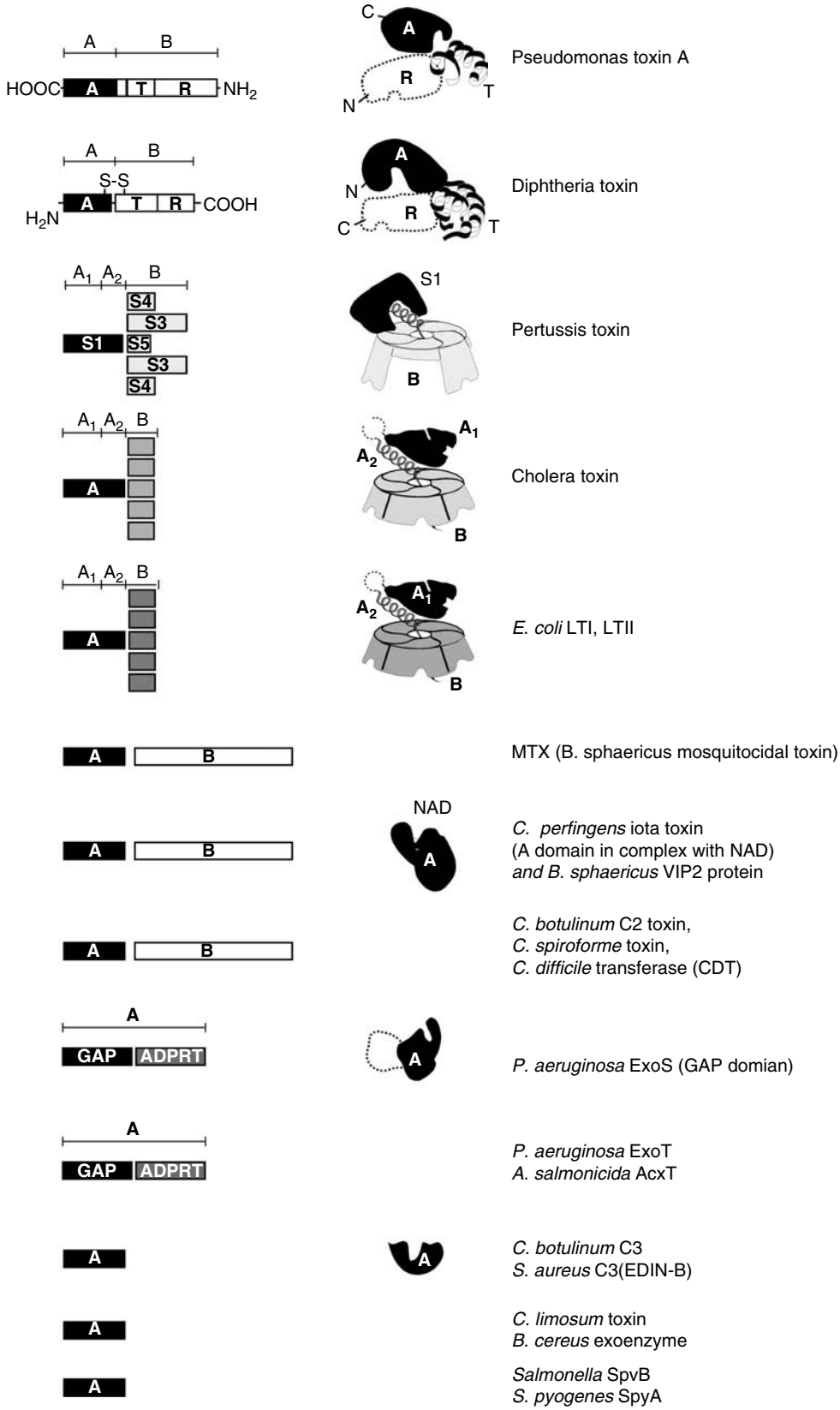



**FIGURE 12.1** Structural organization of bacterial ADP-ribosylating toxins. The enzymically active, toxic moiety (A) is black. The carrier domain(B), when present, is white (left). When crystallographic data are available, a schematic representation of the quaternary structures of the domains is reported (right).

## THE ENZYMATIC REACTION AND THE SUBSTRATES

Two types of ADP-ribosylation reactions are known to occur in nature: poly- and mono-ADP-ribosylation (Ueda and Hayaishis 1985; Althaus and Richter 1987). Poly-ADP-ribosylation occurs mostly in the nucleus of eukaryotic cells where histones and other nuclear proteins are posttranslationally modified first by the covalent attachment of an ADP-ribose group to the carboxyl group of the C-terminal amino acid, and then by the elongation of the ADP-ribose chain through the addition of further ADP-ribose groups. Here, we are interested in mono-ADP-ribosylation, an enzymatic reaction that is mediated by bacterial toxins, phage, and *Escherichia coli* proteins, and a growing family of cytoplasmic and membrane-associated eukaryotic enzymes.

Mono-ADP-ribosyltransferases are produced by bacteriophages, bacteria, and vertebrates (Corda and Di Girolamo, 2003). They bind NAD and transfer the ADP-ribose group to a specific target protein that, following the posttranslational modification, usually changes its function dramatically. The ADP-ribose group is transferred to a nitrogen atom in the side chain of amino acids such as diphthamide, arginine, asparagine, or cysteine, according to the reaction shown in Figure 12.2. Recently, pierisin-1, a novel factor able to ADP-ribosylate DNA, has been discovered in the cabbage butterfly (Kanazawa *et al.*, 2001). Pierisin-1 acts by transferring an ADP-ribosyl group to DNA at N-2 of guanine base and induces apoptosis in mammalian cells accompanied by a release of cytochrome c (Watanabe *et al.*, 2002).

Interestingly, with the exception of actin and of the RNA polymerase, which is the substrate of viral ADP-ribosyltransferases, all the eukaryotic proteins that are ADP-ribosylated by bacterial toxins belong to a quite restricted family: they are GTP-binding proteins (G-proteins). These proteins are molecular switches involved in a variety of cell functions, including cell proliferation, tissue differentiation, signal transduction, protein synthesis, protein translocation, vesicular trafficking, and cytoskeleton structure. These proteins consist of two parts: a common core structure involved in GTP binding, and one or more different domains that act as effectors, or are involved in the interactions with the molecules regulated by these molecular switches (Figure 12.3) (Kjeldgaard *et al.*, 1996). G-proteins have four regions of sequence homology (named Σ1–Σ4 in Figure 12.3) and a structurally conserved core that binds GTP. This domain is the core of the molecular switch that is in the "on" conformation when GTP-bound. Hydrolysis of GTP to GDP causes a conformational change of an α-helix in this core structure that turns the switch in the "off" conformation (Hamm and Gilchrist, 1996; Gilchrist *et al.*, 1998; Sunhara *et al* 1997; Vaughan, 1998; Ji *et al.*, 1998; Gether and Kobilka, 1998; Lefkowitz, 1998). The GTP-binding proteins are divided into four groups: the Ras-like G-proteins, the heterotrimeric G-proteins, the translation factors, and tubulins. Tubulins do not contain the conserved core and are not substrates of ADP-ribosylation. All other groups of G-proteins have one or more components that are substrates of ADP-ribosyltransferases. One question that remains unanswered is whether ADP-ribosylating toxins derive from a common ancestor that recognized the common core structure of G-proteins, or whether during evolution they have independently selected G-proteins as targets many times just because they regulate very important cellular circuits and, therefore, are ideal targets for molecules that intend to intoxicate cells or tissues. The mechanism by which ADP-ribosylating toxins recognize different substrates has so far been open to discussion. However, a recent work reports the identification of several regions that cooperatively define the substrate specificity by exposing a different electrostatic potential at the site of molecular interface (Sun *et al.*, 2004).

The eukaryotic proteins that are mono-ADP-ribosylated by bacterial toxins are the following:

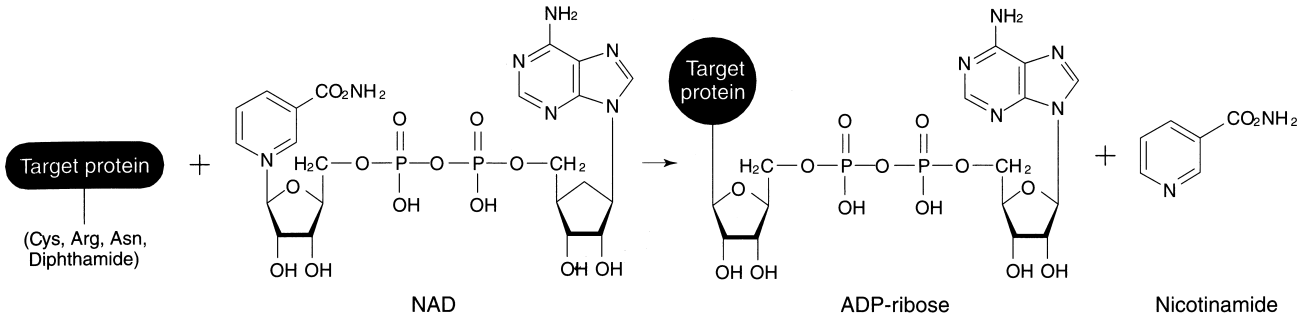



**FIGURE 12.2** Mechanism of the mono-ADP-ribosylation reaction catalyzed by ADP-ribosyltransferases: an ADP-ribose moiety is transferred to a specific target protein and a nicotinamide group is released. Following the post-translational modification the target protein changes its properties.

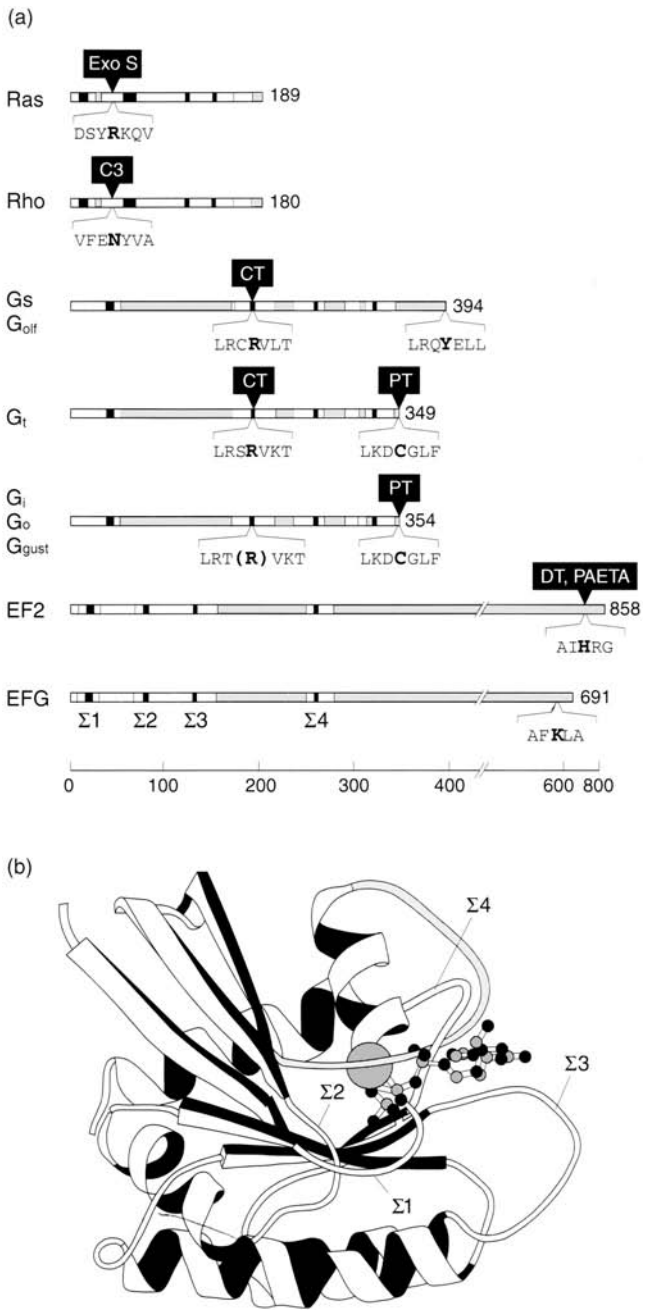



**FIGURE 12.3** (a) schematic representation of GTP-binding proteins. The Σ1–Σ4 are the regions that form the core of the GTP-binding domain. They are present in all G-proteins. In the figure they are indicated by black boxes. The amino acids that are ADP-ribosylated by each toxin are indicated in bold. (b) Three-dimensional structure of the GTP-binding domain common to all G-proteins and formed by the homologous regions Σ1–Σ4.

## Elongation factor 2 (EF2)

Eukaryotic EF2 is a protein of 95,700 Da, which is involved in protein synthesis (Kohno *et al.*, 1986). It contains a posttranscriptionally modified histidine residue (diphthamide 715) (Van Ness *et al.*, 1980a, b), which is ADP-ribosylated by diphtheria toxin DT and Pseudomonas exotoxin A (PAETA) (Honjo *et al.*, 1968; Gill *et al.*, 1969). Following ADP-ribosylation, EF2 is unable to carry out protein synthesis, and this causes rapid cell death. The region containing diphthamide 715 is very close to the anticodon recognition domain of EF2. This suggests that ADP-ribosylation interferes with EF2 binding to the tRNA. The homologous bacterial EFG contains a Lysine in this position, and therefore it is not a substrate for these toxins (see Figure 12.3) (Kohno *et al.*, 1986). Recently, the factor responsible for the first step of the posttranslational modification of EF-2 at $His^{715}$ that yields diphtamide has been identified (Liu and Leppla, 2003). This gene (DESR1) is present in mammalian cells and an analog has also been identified in yeast. Conversely, bacteria, which do not express DESR1, are resistant to diphtheria and pseudomonas toxins. In evolution, the first organisms containing an EF2 that is susceptible to DT and PAETA are the archebacteria.

## Heterotrimeric G-proteins

Heterotrimeric G-proteins are involved in transduction of signals from surface-exposed receptors to intracellular effectors. They generally consist of an α subunit containing the GTP-binding domain and the intrinsic GTPase activity and the βγ subunit complex, containing two protein chains. In the "off " position, the α subunit binds ADP and is found in a membrane-bound complex together with the β and γ subunits. A positive signal from the receptor will cause the exchange of the GDP with GTP. The GTP-bound form of α dissociates from the βγ complex and interacts with a downstream effector molecule, thus transducing an extracellular event in a change of the intracellular chemical environment. They usually regulate the activity of enzymes such as adenylate cyclase, phospholipase C, and cyclic GMP-phosphodiesterase, which release secondary messengers into the cytoplasm as a response to external stimuli (Stryer and Bourne, 1986; Neer and Clapham, 1988). Adenyl cyclase, for instance, is regulated by two GTP-binding proteins: $G_s$ and $G_i$. $G_s$ receives signals from stimulatory receptors located on the surface of eukaryotic cells and stimulates the activity of adenyl cyclase. $G_i$, on the other hand, receives signals from the inhibitory receptors and inhibits the adenyl cyclase activity

(Gilman, 1984). In addition to $G_s$ and $G_i$, the family of heterotrimeric G proteins contains $G_o$, $G_t$, $G_g$, $G_{olf}$, and many other less characterized proteins with similar function. As shown in Figure 12.3, cholera toxin ADP-ribosylates Arg201 of the α subunit of $G_s$, $G_{olf}$, $G_t$. The corresponding Arg residues in $G_i$ and $G_o$ are ADP-ribosylated only when they are activated by the receptor. Arg201 is located in the core region that is involved in binding GTP. ADP-ribosylation has therefore a direct consequence on the enzymatic activity: it slows down GTP hydrolysis, thus keeping $G_s$ in a permanent "on" position. Pertussis toxin ADP-ribosylates Cys352 of the α subunit of $G_i$, $G_o$, $G_{gust}$, $G_t$, and other G proteins, but is unable to ADP-ribosylate $G_s$ and $G_{olf}$ that have a Tyrosine instead of a Cysteine in this position. This carboxyterminal region of Gα is involved in interaction with the receptor, and therefore ADP-ribosylation causes receptor uncoupling. ADP-ribosylation of G proteins by cholera and pertussis toxins causes a variety of effects in different tissues. In the case of adenyl cyclase, treatment with cholera toxin causes constitutive activation of the enzyme and accumulation of the second messenger cAMP, while treatment with pertussis toxin uncouples $G_i$ from its receptor so that it becomes unable to inactivate adenyl cyclase.

## Ras, Rho, and the small GTP-binding proteins

The Ras proteins are a large family of membrane-associated, GTP-binding proteins of approximately 21 kDa. They function in transmembrane signaling systems that control a variety of cellular processes including growth, proliferation, and differentiation of cells. The human Ras family consists of three highly homologous members: N-, K-, and K-Ras. They contain a "CAAX" carboxyterminal box that can be modified by the addition of a farnesyl lipid moiety, thus increasing their hydrophobicity. In the active conformation, the GTP-bound Ras interacts with the GTPase activating proteins (GAPs) effector molecules that turn it off by stimulating GTP hydrolysis. Many Ras proteins are known to be protooncogenes, requiring a single amino acid change to turn them into oncogenes. The mutations that are mostly found in tumors are in position 12 in Σ1 and in position 61 in Σ3. The oncogenic proteins exhibit a low GTPase activity following interaction with GAP, and therefore are blocked mostly in the "on" position.

Rho (Ras homology) are a subgroup of the Ras family GTPases containing several members including RhoA, B, C, D, E, and G, Rac1, 2, and 3, Cdc42, etc. Like Ras they contain the "CAAX" carboxyterminal box and can be posttranslationally modified. However, while Ras is only plasma membrane-associated, Rho can be found in a variety of locations, including the cytoplasm (RhoA and C, Rac1 and 2), the endosomes (RhoB), or the Golgi and endoplasmic reticulum (Cdc42). Rho proteins are controlled by three groups of regulatory proteins. Guanine nucleotide exchange factors (GEFs) induce activation of Rho by facilitating the GDP/GTP exchange, GTPase-activating proteins (GAPs) stimulate the hydrolysis of GTP thus turning off Rho proteins, while Guanine dissociation inhibitors (GDIs) keep Rho in the inactive form. Rho, Rac, and Cdc42 are involved in the regulation of the actin cytoskeleton. Rho induces formation of focal adhesion and actin stress fibers. Rac is involved in lamellopodia formation and induces adhesion complexes. Cdc42 induces the formation of microspikes. In summary, the Rho family of GTPases are involved in focal adhesion, integrin function, cell movement, and cell division. Ras is ADP-ribosylated at Arg41 by the Exoenzyme S of *P. aeruginosa*, while Rho is modified at Asn41 by the C3 enzyme of *C. botulinum* and *C. limosum*, by the *Staphylococcus aureus* EDIN protein, and by the VIP2 of *Bacillus cereus*. ADP-ribosylation causes inactivation of Ras and Rho, inability to stimulate actin polymerization with consequent loss of cell shape and cell rounding.

## Actin

Actin, a polypetide of 375 amino acid residues, is an ATP-binding protein that, in addition to contraction of muscle cells, in non-muscle cells controls the shape and the spatial organization of the cells, the cell movement, endo- and exocytosis, vesicle transport, cell contact, and mitosis. Microfilaments, which are the major structure of the cytoskeleton, are filamentous structures of 7–9 nm diameter, composed by polymerized actin. Rapid changes in cell shape are based on the ability of microfilaments to polymerize and depolymerize. Several bacterial toxins ADP-ribosylate monomeric actin at Arg 177. This Arg is located at the contact site between actin monomers, and therefore it can be modified in monomeric G actin, but it is not available for ADP-ribosylation in polymerized actin. Once ADP-ribosylated, monomeric actin is unable to make contacts with the other actin monomers, and therefore it cannot polymerize. As a consequence, toxin-treated cells are not able to build microfilaments and their cytoskeletal structure is rapidly destroyed. The toxins that ADP-ribosylate actin are *C. botulinum* C2 toxin, *Clostridium perfringens* iota toxin, *Salmonella enterica* SpvB toxin, and the ADP-ribosylating toxins of *Clostridium spiroforme* and *Clostridium difficile*.

## CELL ENTRY

Since the target proteins of ADP-ribosylating toxins are all located in the cytosol or in the inner face of the cytoplasmic membrane, the toxins need to cross the cell membrane in order to reach their intracellular targets. This is done mainly in two ways: (i) receptor-mediated endocytosis (see Figure 12.4, paths 1 and 2); or, (ii) direct injection of the toxin from the bacterium into the cytosol of the eukaryotic cell by a type III secretion system (see Figure 12.4, path 3).

### Receptor-mediated endocytosis

Receptor-mediated endocytosis is used by soluble toxins with an A/B structure and by binary toxins where the A and B domains are physically separated. The toxins with this structure are released by bacteria in the culture supernatant and bind to the surface of eukaryotic cells by their B domain, which contains a receptor-binding site. Following binding, the A/B toxins are internalized and located in membrane-bound vesicles (early endosomes). In the case of binary toxins, the B domain binds first to the cell receptor and then captures the A domain to the cell surface. The binding components of C2 toxin and iota toxin have been shown to form heptamers in solution (Barth *et al.*, 2000; Blocker *et al.*, 2001). In the case of C2, once formed, the heptamers insert as pores into the lipid bilayer and mediate the translocation of the active subunit into the cytosol (Blocker *et al.*, 2003). Furthermore, the host cell chaperone Hsp90 has recently been shown to be essential in the second step of translocation of C2I and iota toxin from endosomes into the cytosol (Haug *et al.*, 2004). Following internalization, two quite different pathways are used by different toxins to translocate their A domain into the cytosol. The two pathways have been best studied for diphtheria and cholera toxin, respectively. Diphtheria toxin A domain crosses the membrane early after internalization (see Figure 12.4, path 1). As soon as the pH of the endosomes decreases to 5.5 units, the B domain changes conformation and exposes hydrophobic α-helices that are not soluble any longer in water and therefore penetrate the lipid bilayer of the membrane. This initiates a process that favors the translocation of the A subunit across the membrane and also involves the action of a cytosolic translocation factor (TFC) complex. (See the paragraph on diphtheria toxin for a more detailed description of this process.)

The toxins known to cross the membrane by a mechanism similar to diphtheria toxin are botulinum and tetanus toxins.

Conversely, cholera toxin has a much more complicated intracellular route before it reaches the cytoplasm (Figure 12.4, path 2). Following internalization in early endosomes, it undergoes a retrograde transport back to the Golgi apparatus, across it until it reaches the endoplasmic reticulum (ER). The routing of the A subunit to this pathway is believed to be medi-

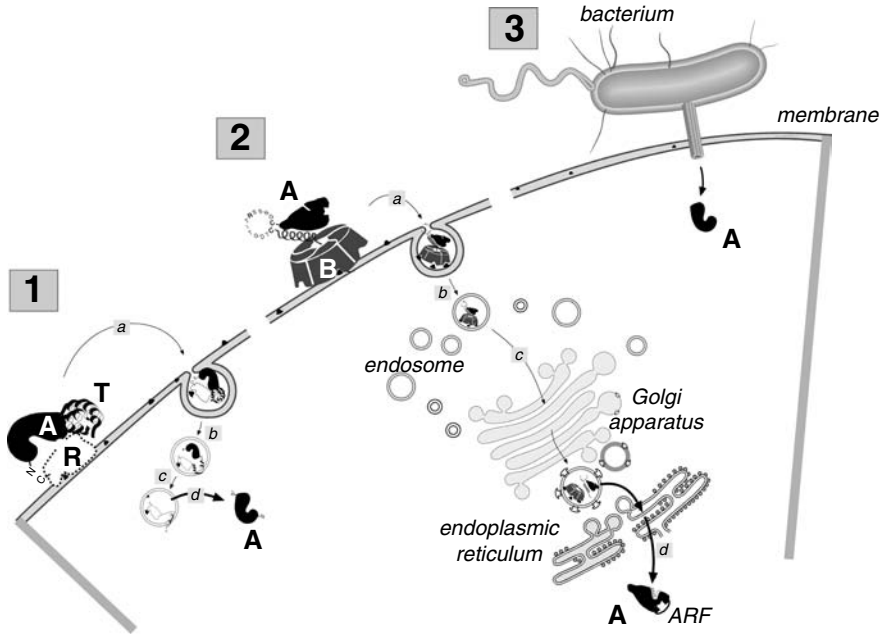



**FIGURE 12.4** Schematic representation of the three strategies used by ADP-ribosylating toxins to enter into cells.

ated by an amino acid motif (KDEL), which is similar to the endoplasmic reticulum retention domain of eukaryotic proteins. Once in the ER, the misfolded catalytic toxin subunit uses a retro-translocation path to finally reach the cytosolic compartment, where it rapidly refolds, avoids the proteasome, and induces toxicity (Lencer and Tsai, 2003).

The toxins that follow the intracellular route of cholera toxin are: Shiga toxin, the related verotoxin, pertussis toxin, *E. coli* heat-labile enterotoxin, and Pseudomonas exotoxin A (Johannes and Goud, 1998).

### Direct transfer of toxins from bacteria to eukaryotic cells

Some bacteria have evolved sophisticated transport systems to transfer their weapons directly into eukaryotic cells. The most famous are the type III and type IV secretion systems of Gram-negative bacteria. In these cases, the transmembrane machinery of flagella and conjugative pili, respectively, were evolved into contact-dependent delivery systems able to translocate macromolecules into eukaryotic cells. The toxins translocated by these mechanisms do not need a B domain since they are directly injected into eukaryotic cells (Figure 12.4, path 3). ADP-ribosylating toxins known to be injected into cells by a type III secretion system are Exoenzyme S and its homologue ExoT of *P. aeruginosa* (Sundin *et al.*, 2004). This is made by a single polypeptide chain containing the enzymatically active portion in the carboxyterminal region and a part recognized by the secretion system in the aminoterminal domain. Furthermore, the ADP-ribosyltransferase AexT produced by the fish pathogen *Aeromonas salmonicida* has also been shown to be secreted by a type III secretion system (Burr *et al.*, 2003).

A further example of a toxin secreted by specialized systems is pertussis toxin (PT). This protein is exported via a two-step process. In the first step, the individual subunits are secreted into the periplasm by a Sec-dependent mechanism. Upon assembling of the components to reconstitute the holotoxin, pertussis toxin traverses the outer membrane with the assistance of the type IV *Ptl* secretion system (Weiss *et al.*, 1993; Farizo *et al.*, 2000).

## BACTERIAL TOXINS

### Toxins acting on protein synthesis

#### *Diphtheria toxin*

Diphtheria toxin is a protein molecule, of 58,350 Da, that is released into the supernatant by toxinogenic *Corynebacterium diphtheriae* strains (Pappenheimer, 1977; Collier, 1982). The toxin is synthesized as a single polypeptide chain that, after mild trypsin treatment and reduction of a disulfide bond, can be divided into two functionally different moieties: fragment A and fragment B of 21,150 and 37,200 Da, respectively (Pappenheimer, 1977) (Figure 12.5A). The molecule contains four cysteines and two disulfide bridges: the first one joins Cys-186 to Cys-201 and links fragment A to fragment B, whereas the other is contained within fragment B and joins Cys-461 to Cys-471. The determination of the three-dimensional structure of the molecule (Choe *et al.*, 1992; Bennett *et al.*, 1994) has shown that the toxin can be divided into three separate domains: the C (catalytic) domain, corresponding to fragment A; the T (transmembrane or translocation) domain, composed of nine α-helices (TH1–TH9) (Figure 12.5C); and the carboxyterminal R (receptor-binding) domain.

Fragment A is a $NAD^+$ -binding enzyme that catalyzes the transfer of the ADP-ribosyl group to a posttranslationally modified histidine residue (diphthamide) present in the cytoplasmic elongation factor 2 (EF-2) of eukaryotic cells (Brown and Bodley, 1979; Van Ness *et al.*, 1980a). The EF-2-ADP-ribose complex is inactive, and therefore diphtheria toxin causes inhibition of protein synthesis and cell death. It has been shown that *in vitro* a single molecule of fragment A is enough to kill one eukaryotic cell (Yamaizumi *et al.*, 1978). This observation correlates well with the *in vivo* studies that show that, on a weight basis, diphtheria toxin is one of the most potent bacterial toxins, the minimal lethal dose of which is below 0.1 $\mu$g/kg of body weight (Pappenheimer, 1984).

Although the entire lethal activity of diphtheria toxin is located in fragment A, fragment B is required for receptor-binding and for translocation of fragment A across the cell membrane. The COOH-terminal domain involved in receptor-binding (R) and the NH2-terminal hydrophobic domain (T), involved in the interaction with the cell membrane, mediate this process. The receptor is the heparin-binding, EGF-like growth factor precursor (Naglich *et al.*, 1992; Hooper and Eidels, 1995; Brown *et al.*, 1993; Iwamoto *et al.*, 1994) that is present on most animal cells. Murine cells contain amino acid changes in the receptor that make rodents insensitive to diphtheria toxin (Mitamura *et al.*, 1995; Brooke *et al.*, 1998). Following receptor-binding, DT is internalized by receptor-mediated endocytosis. When the pH of the endosomes decreases below pH 5.5, fragment B undergoes a conformational change that exposes the hydrophobic regions of the T domain, allowing interaction with the endosomal membrane and translocation of denaturated fragment A from the endosome into the cytoplasm (Sandvig and Olsnes,

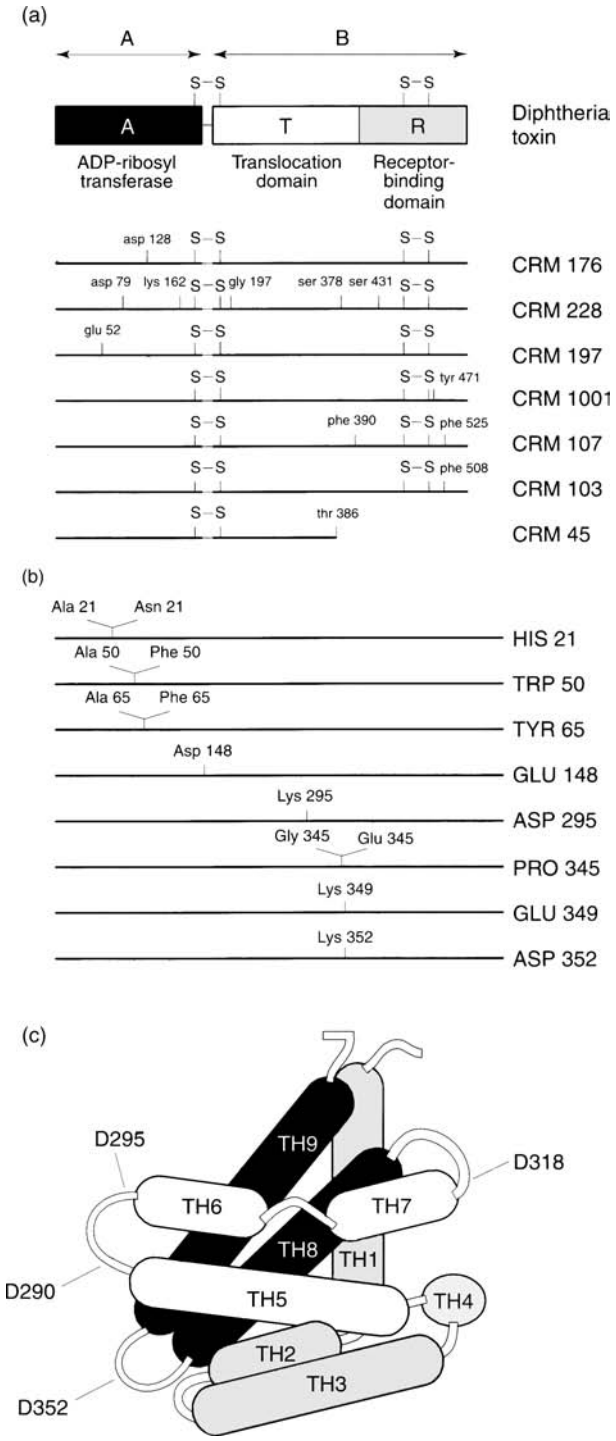



**FIGURE 12.5** Diphtheria toxin: (a) structure of the toxin and mutant CRMs obtained by random chemical mutagenesis; (b) mutants obtained by site-directed mutagenesis; (c) three-dimensional structure of the seven α-helices that form the hydrophilic T domain. The position of the mutations described in this region are shown.

1981; Cieplak *et al*., 1987; Neville and Hudson 1986; Papini *et al*., 1987a; Papini *et al*., 1987b). Therefore, whereas fragment A is very stable over a wide range of pH, temperature, and denaturing agents (it can be boiled, treated with urea, or various detergents without affecting its enzymatic activity), fragment B is very sensitive to low pH and is unstable in many buffers. According to a recent model, the A subunit of DT is able to cross the endosomal membrane making use of a metastable transmembrane domain, which has also been identified (Wolff *et al*., 2004). Although much is known on the mechanisms of receptor-binding and receptor-mediated endocytosis of native DT, little is known about the precise mechanisms that drive the translocation of the active subunit across endosomal membranes. Unfolding of the A domain before translocation was initially demonstrated by Wiedlocha *et al.*, (1992) and by Falnes *et al.*, (1994). Ratts and colleagues (2003) have shown that *in vitro*, a cytosolic translocation factor (CTF) complex, composed of Hsp90 and thioredoxin reductase, is essential for the translocation and cytoplasmic release of the A domain from early endosomes. Another activity described for DT is apoptosis of target cells. This activity is apparently mediated by the A fragment, but is not linked to the enzymatic activity. In fact, apoptosis has also been described for cross-reacting material (CRM197), an enzymatically inactive, nontoxic mutant of DT (described below). Whether apoptosis plays a role in toxicity *in vivo* is unclear; however, it cannot be a major role because the mutants, which are active in apoptosis but enzymatically inactive, are non-toxic *in vitro* and *in vivo*.

The diphtheria toxin gene is carried by a family of closely related bacteriophages (corynebacteriophages) that are able to integrate into the bacterial chromosome and convert non-toxinogenic, non-virulent *C. diphtheriae* strains into toxinogenic, highly virulent species (Freeman, 1951; Uchida *et al*., 1971). Lysogenic *C. diphtheriae* strains produce diphtheria toxin only when the medium is depleted of iron. The iron regulation of DT synthesis was discovered by A.M. Pappenheimer (1938) and is today well understood at the molecular level. The region upstream of the DT gene contains a sequence that is bound by an iron-binding repressor that dissociates from DNA only in the absence of iron (Qiu *et al*., 1995; Ding *at al*., 1996; Pohl *et al*., 1998; White *et al.*, 1998).

The best-known toxinogenic corynephages are βtox+ (Costa *et al*., 1981), isolated in 1950 during an outbreak of diphtheria in Canada, and ωtox+, isolated from the hypertoxinogenic PW8 strain (Rappuoli *et al*., 1983a). The process of integration of corynephage DNA into the bacterial chromosome closely resembles the mechanism of lysogenization described by Campbell (1962)

for bacteriophages: the phage DNA contains a locus called *phage attachment site* (*att*P), which is homologous to a sequence in the bacterial chromosome (bacterial attachment site or *att*B). Following site-specific recombination between these two sites, the phage DNA becomes stably integrated into the bacterial chromosome. Unlike *E. coli,* the chromosome of *C. diphtheriae* contains two primary attachment sites (attBl and attB2) (Rappuoli *et al*., 1983b; Rappuoli and Ratti, 1984). This allows the stable integration of two copies of corynephage DNA into the bacterial chromosome (Rappuoli *et al*., 1983b). Because each phage carries one copy of the *tox* gene, double lysogens produce twice the toxin produced by monolysogens. This property has been very important for obtaining strains hyperproducing CRM197, a nontoxic form of diphtheria toxin, that is used as a carrier for *Haemophilus influenzae* type B (Anderson *et al*., 1987; Egan *et al*., 1995; Rothbrock *et al*., 1995) and for meningococcal (Costantino *et al*., 1992) conjugate vaccines, and has been proposed as a new vaccine against diphtheria (Rappuoli, 1983).

The gene coding for diphtheria toxin is contained within an 1850 base pair (bp) *EcoRI-HindlII* DNA fragment, which has been cloned and entirely sequenced from phages ßtox+ and ωtox+ (Greenfield *et al*., 1983; Ratti *et al*., 1983). The sequences of the two *tox* genes obtained from phages isolated more than 50 years apart are identical, showing the remarkable conservation of the diphtheria toxin molecule. With the determination of the x-ray structure of DT in complex with its substrate NAD, DT was the first toxin for which structure-function relationships were elucidated, and it has been a model for all the other toxins (Bell and Eisenberg, 1997).

### *Mutants of diphtheria toxin*

Most of the functional and structural properties of DT were initially deduced from the analysis of a number of non-toxic DT mutants (cross-reacting materials of CRMs), encoded by corynephages that had been mutagenized by nitroguanidine (Uchida *et al*., 1971, Uchida *et al*., 1973a, b, c; Laird and Groman, 1976). The analysis of the properties of these mutants and the sequence of their genes was the first tool to map the functional domains of diphtheria toxin, and to identify some of the amino acids that play an important role in the enzymatic activity of fragment A, the receptor-binding domain of fragment B, and the translocation of fragment A across the eukaryotic cell membrane. The most relevant diphtheria toxin mutants are described below and reported in Figure 12.5a.

CRM 176 contains a Gly-128 to aspartic acid mutation that reduces the enzymatic activity of fragment A by a factor of 10 (Comanducci *et al*., 1987). CRM 228 contains two mutations in fragment A and three in fragment B, which result in inactive A and B fragments (Kaczorek *et al*., 1983). The mutation Gly79 → Asp has been later shown to be golely responsible for the lack of enzymatic activity of fragment A (Johnson and Nicholls, 1994a).

CRM 197 contains a single Glycine to Glutamic acid change in position 52 that makes the fragment A unable to bind NAD+ and, therefore, enzymatically inactive (Giannini *et al*., 1984; Pappenheimer *et al*., 1972). Being enzymatically inactive, and therefore non-toxic, but otherwise identical with diphtheria toxin, CRM 197 has been proposed as a natural candidate to develop a new vaccine against diphtheria. However, some subtle structural differences between diphtheria toxin and CRM 197 have been detected (Bigio *et al*., 1987), making this mutant more susceptible to proteases and less immunogenic than diphtheria toxoid (Pappenheimer *et al*., 1972). After stabilization with formalin, CRM 197 becomes immunogenic and able to induce protective antibody titers against diphtheria (Porro *et al*., 1980); however, its potency per μg of protein is still slightly lower than that of diphtheria toxoid. However, both DT and its nontoxic mutant CRM197 have found wide application as carrier molecules for glycoconjugate vaccines.

CRM 1001 has an enzymatically active fragment A and a fragment B that binds the toxin receptors, but is unable to translocate fragment A across the eukaryotic cell membrane. The nucleotide sequence of its gene has shown that Cys-471 has been replaced by Tyrosine, thus inhibiting the formation of the disulfide bridge between Cys-461 and Cys-471 (Dell'Arciprete *et al*., 1988).

CRM 103 and CRM 107 are two mutants that are unable to bind the receptors on the surface of eukaryotic cells (Laird and Groman, 1976; Greenfield *et al*., 1987). Sequence analysis has shown that both molecules contain mutations in the R domain. In CRM 103, Ser-508 has been changed to Phenylalanine, whereas in CRM 107 both Leu-390 and Ser-525 have been replaced by a Phenylalanine. CRM 45 is a shorter molecule prematurely terminated because of a nonsense mutation that introduces a stop codon at position 387; therefore, CRM 45 lacks entirely the R domain and is unable to bind the toxin receptors (Giannini *et al*., 1984).

The characterization of the structure-function relationships, initially performed using the above mutants produced by nitrosoguanidine mutagenesis, has made great progress by studying mutant proteins constructed through site-directed mutagenesis, and containing amino acid changes in those positions that the crystal structure and biochemical studies had suggested to be important. Among the mutations produced by site-directed mutagenesis, the most relevant are described below and shown in Figure 12.5.

Histidine 21, initially identified as important for catalysis by biochemical methods (Papini *et al*., 1989), was later confirmed to be so important that it cannot be substituted with other amino acids without abolishing the enzymatic activity. Some activity was only maintained when His was replaced by Asn (Johnson *et al*., 1994b; Blanke *et al*., 1994a).

Substitution of Tryptophan 50 with Alanine decreased ADP-ribosyltransferase by $10^5$-fold, while substitution with Phenylalanine had only minimal effects, suggesting an important role for an aromatic residue in this position for NAD affinity (Wilson *et al*., 1994).

Tyrosine 65 was initially identified as important for binding the nicotinamide ring by photolabeling studies (Papini *et al*., 1991), and later confirmed to be relevant by mutagenesis studies. Alanine substitution caused a 350-fold decrease of enzymatic activity, while Phenylalanine substitution caused only a small decrease (Blanke *et al*., 1994b).

Glutamic acid 148 was also initially identified as being near the catalytic site by photoaffinity labeling (Carroll *et al*., 1985), and was later shown to be so important for catalysis that it could not be substituted even with the closely related Aspartic acid (Tweten *et al*., 1985).

Asp 295, which is located in the hairpin loop between the α-helices TH5 and TH6 of the T domain, was shown to be important for membrane interaction of fragment B, because its substitution with a Lysine residue caused reduction of toxicity (Silverman *et al*., 1994). Finally, several amino acids, located between α-helices TH8 and TH9, were also found to be very important for DT translocation across the endosomal membrane. Substitution of Proline 345 with Glycine or Glutamic acid caused 99% reduction of toxicity (Johnson *et al*., 1993). Substitution of Glutamic acid 349, or Aspartic acid 352 with Lysine, caused more than 100-fold reduction of toxicity (Silverman *et al*., 1994).

The information deriving from biochemical, structural, and mutagenesis studies has increased our knowledge about the way the catalytic and translocation domains work. The present understanding is described below.

The active site (Figure 12.11) is formed by an α-helix bent over a β-strand, which forms the ceiling and the floor of the NAD-binding cavity, respectively. Within the cavity, Tyrosine 52 and Tyrosine 65 are parallel and between their aromatic rings there is just enough space to allow the entrance of the nicotinamide ring of NAD, which is then held firmly sandwiched between the two Tyrosines. At each side of the NAD-binding cavity, there are two amino acids that are essential for catalysis: His 21 and Glu 148.

His 21 is likely to be involved in positioning the NAD into the cavity, so that it is available for the nucleophilic attack of the substrate. Glu 148 is likely to be involved in interaction with the incoming substrate molecule. The structure of the active site shown in Figure 12.11 is conserved in all ADP-ribosylating enzymes known to date (Domenighini *et al*., 1991; Domenighini *et al.,* 1994; Domenighini *et al*., 1995a).

The T domain (Figure 12.5C) is formed by nine α-helices arranged in three layers. The internal one contains helices TH8 and TH9 that are hydrophobic and amphipathic, respectively. The intermediate one contains helices TH5, TH6, and TH7, which form a second hydrophobic shell. Finally, the external layer contains helices TH1, TH2, and TH3, which are rich in charged residues and help in maintaining the T domain and the toxin in a soluble form at neutral pH. When the pH decreases within the endosomes during toxin internalization, the charged residues located at the hairpin between two hydrophobic α-helices, such as Glu 295, Glu 349, and Asp 352, lose their charge, thus making the entire structure hydrophobic. This triggers the interaction of the T domain with the membrane and the consequent translocation of the A domain in the cytoplasmic site.

The genetic organization of DT, with the region encoding for the receptor-binding domain at the 3'-end of the gene, has allowed the development of a number of hybrid toxins in which the receptor-binding domain has been replaced by interleukin 2, or by the melanocyte hormone MSH (Murphy *et al*., 1986; Williams *et al.,* 1987; Bacha *et al*., 1988). These new molecules kill activated T cells and MSH receptor-bearing cells, respectively. They might find therapeutic applications for the treatment of allograft rejection and melanomas.

### *Pseudomonas exotoxin A*

*Pseudomonas* exotoxin A (PAETA) is secreted in the culture medium as a single polypeptide chain of 613 amino acids of which the sequence and the three-dimensional structure at 3 Å of resolution is known (Gray *et al*., 1984; Allured *et al*., 1986). According to X-ray crystallography studies, the molecule can be divided into three domains (Hwang *et al*., 1987; Siegall *et al*., 1989; Pastan and FitzGerald, 1989). Domain I is composed of two non-contiguous regions: Ia comprising amino acids 1–252 and Ib composed of amino acids 365–404.

Domain II is composed of amino acids 253–364, while domain III comprises amino acids 405–613 (see Figures 12.1 and 12.6). Four disulphide bridges are present, two located in domain Ia, one in domain Ib, and one in domain II. Genetic studies, based mainly on the expression of mutated forms of the PAETA gene in *E. coli*, have shown that the deletion of domain Ia results in non-toxic, enzymatically active molecules

that cannot bind the cells. A similar result can be obtained by mutating Lys57 to Glu (Jinno *et al*., 1988). Deletions in domain II result in molecules that bind to the cells and that are enzymatically active but not toxic. A similar result can be obtained by mutagenizing Arg276, Arg279, and Arg230 (Jinno *et al*., 1989) or by converting Cys265 and Cys268 to other amino acids, thus changing the structure of domain II (Siegall *et al*., 1989). Deletions or mutations in domain III result in enzymatically inactive molecules (Siegall *et al*., 1989).

Based on these observations, it can be concluded that the three domains observed in the X-ray structure correspond exactly to three functional domains involved in cell recognition (domain I), membrane translocation (domain II), and ADP-ribosylation of elongation factor 2 (domain III). To become active, the PAETA toxin requires an intracellular, furin-mediated proteolytic cleavage to generate a 37 kDa C-terminal fragment that is then translocated to the cytoplasm to reach the EF2 target (Chiron *et al.*, 1997). By using a fluorescence resonance energy transfer approach, the mechanism of interaction between PAETA and its substrate EF has been studied, showing that the binding is strongly dependent on the pH. Furthermore, the finding that EF-2 bound to GDP or GTP is still recognized by PAETA shows how adaptable this toxin is in ADP-ribosylating its substrate (Armstrong *et al.*, 2002). In particular, mutational analysis affecting the last five residues at the carboxy-terminus of the enzymatic domain resulted in complete loss of cytotoxicity; this segment (Arg-Glu-Asp-Leu-Lys, REDLK) closely resembles the KDEL motif that is a well-defined endoplasmic reticulum retention sequence and that has also been found at the C-terminus of other ADP-ribosyltransferases, such as cholera toxin and heat-labile enterotoxin of *E. coli* (Chaudhary *et al.*, 1990). It has been postulated that the sequence REDLK may be a recognition signal required for entry of the ADP-ribosylation domain of PAETA into the cytosol.

PAETA can be described as a typical bacterial toxin with an A-B structure, having a mechanism of action identical to diphtheria toxin. Similarly to DT, PAETA has also been shown to induce apoptosis in human mast cells by a caspase-3 and -8 mediated mechanism (Jenkins *et al.*, 2004). Although identical in function, DT and PAETA have an opposite structural organization: the enzymatically active domain is at the C-terminus of PAETA and at the N-terminus of DT. While the B domains of DT and PAETA do not have any structural similarity, the enzymes share a common structure of the catalytic site (see Figure 12.11 for details) (Brandhuber *et al*., 1988; Carroll and Collier, 1988). The structure of this site, well-described by X-ray crystallography, has been further corroborated by functional studies, which have identified amino acids playing a key role in enzymatic activity. Glu553 was initially shown to be at the catalytic site, because it was the only amino acid photoaffinity-labeled by NAD+ (Carroll and Collier, 1987). Later it was shown that substitution of Glu553 with any amino acid, including Asp, decreased the enzymatic activity by a factor of 1,000 (Douglas and Collier, 1990), and that deletion of Glu553 completely abolished the toxicity of PAETA. Similarly, iodination of Tyr481, which is also at the catalytic site, was shown to abolish enzymatic activity (Brandhuber *et al*., 1988). Other amino acids that, although not located at the catalytic site, have been shown to be essential for enzymatic activity are His 426 and residues 405–408 (Galloway *et al*., 1989). His 426 has been proposed to be necessary for the interaction between PAETA and EF2.

The well-defined structural divisions in separated domains make PAETA an ideal candidate for the development of chimeric toxins by replacing the gene parts encoding for domain I with others encoding for cell-binding domains with different specificities. So far, nucleotides encoding domain I have been replaced by sequences encoding interleukin 2, interleukin 6, interleukin 4, and T cell antigen CD4. In all instances, expression of these genes in *E. coli* has given new toxins, which specifically kill the cells bearing the receptor recognized by the new domain I. Such molecules are promising candidates for the treatment of arthritis and

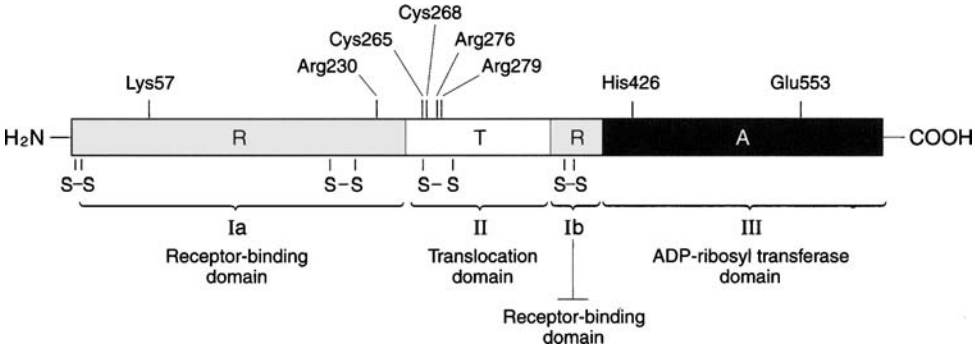



**FIGURE 12.6** Domain organization of *Pseudomonas aeruginosa* exotoxin A (PAETA). The enzymic domain (A) is shown in black, while the receptor-binding (R, Ia and Ib) and the translocation (T) domains are colored grey and white, respectively. The more relevant mutants affecting either cell binding or enzymic activity are reported in the upper portion of the figure. Disulfide bridges are also shown.

allograft rejection (PAETA-IL2), AIDS (PAETA-CD4), and other diseases (Chaudhary *et al*., 1987; Chaudhary *et al*., 1988; Siegall *et al*., 1988; Lorberboum-Galski *et al*., 1988; Ogata *et al*., 1989; Baldwin *et al*., 1996; Mori *et al*., 1997; Zimmermann *et al*., 1997; Essand *et al*., 1998).

## Toxins acting on signal transduction

### *Acting on large G proteins*

*Pertussis toxin*

Pertussis toxin (PT) is a protein of 105,000 Da released into the extracellular medium by *Bordetella pertussis*, the etiological agent of whooping cough. PT is a complex bacterial toxin composed of five different subunits that have been named S1 (21 220 Da), S2 (21 920 Da), S3 (21 860 Da), S4 (12 060 Da), and S5 (11 770), according to their electrophoretic mobility (Tamura *et al*., 1982; Sekura *et al*., 1985). Exposure of PT to 2 M urea disassembles the PT into the monomer A (subunit S1) and the oligomer B (which comprises the subunits S2, S3, S4, and S5). Upon exposure to 5 M urea, the B oligomer can be dissociated into two dimers: dimer 1 (comprising S2 and S4) and dimer 2 (comprising S3 and S4), and the monomer S5 (see Figure 12.1). With 8 M urea, PT dissociates into five monomeric subunits (Tamura *et al*., 1982).

As in the case of the other ADP-ribosylating toxins, the B oligomer of PT binds unidentified glycoconjugate receptors on the surface of eukaryotic cells and allows the toxic subunit S1 to reach its intracellular target proteins. In Chinese hamster ovary (CHO) cells, the PT receptor has been shown to be a 165-kDa glycoprotein, which binds the PT B oligomer through a branched mannose structure containing sialic acid. Dimers S2–S4 and S3–S4 are also able to bind the same receptor (Witvliet *et al*., 1989; Stein *et al*., 1994a).

S1 ADP-ribosylates the Cys353 of the α subunit of protein $G_i$, and the corresponding cysteine in $G_o$, and transducin (see Figure 12.3) (Katada *et al*., 1983; West *et al*., 1985). Gsα, which contains a Tyr in place of the Cys residue, is not ADP-ribosylated by PT. ADP-ribosylation of the above G proteins causes alteration in the response of eukaryotic cells to exogenous stimuli and results in a variety of phenotypes. *In vivo*, the most relevant consequences of PT intoxication are leukocytosis, histamine sensitization, increased insulin production with consequent hypoglycemia, and potentiation of anaphylaxis (Sekura *et al*., 1985). Although its direct role in the severe coughing disease has long been uncertain, data from a recent study indicate that PT plays an early role in the colonization by *B. pertussis* and that its enzymatic activity is required for full colonization (Carbonetti *et al.*, 2003). *In vitro*, PT has a number of different activities, the most relevant of which is the change in cell morphology in CHO cells, a phenotype which is able to detect as little as 10 pg of active PT (Hewlett *et al*., 1983). Further *in vitro* activities are hemagglutination, T-cell mitogenicity, inhibition of migration of peritoneal macrophages, enhancement of receptor-mediated accumulation of cAMP, and many others (Sekura *et al*., 1985). In contrast to the other bacterial toxins where all the activities are mediated by the enzymatically active subunit, in the case of PT several biological activities are due to the B oligomer (Tamura *et al*., 1983). In fact, the B subunit of pertussis toxin is a polyclonal mitogen for T cells. *In vitro* the effect requires at least 0.3 μg of pertussis toxin or of its B subunit. The effect has not been observed *in vivo*, possibly because the dose required for activity is never achieved. It also has hemagglutination activity. *In vitro*, the effect requires 0.3 μg/ml of wild-type PT, or of its B subunit. In addition, the B oligomer is able to induce signal transduction through inositole phosphate pathway. The numerous *in vitro* and *in vivo* activities of PT are listed in Table 12.1. As shown in the table, the toxic activities are all abolished when the active site is inactivated by site-directed mutagenesis, such as mutant PT-9K/129G. On the other hand, the non-toxic biological activities that are mediated by the receptor-binding site present in the B oligomer are all maintained, even in the absence of ADP-ribosyltransferase activity.

The genes encoding for the five subunits of pertussis toxin are clustered in a fragment of DNA of 3,200 base pairs, organized in an operon structure and in the following order: S1, S2, S5, S4, and S3 (Nicosia *et al*., 1986; Locht and Keith, 1986). Each of the five subunits are co-translationally exported into the periplasmic space where the holotoxin is assembled. The release of the toxin into the extracellular medium requires the products of the *ptl* locus, an operon containing 11 genes, which is homologous to the *tra* operon of *E. coli* and the VirB operon of *Agrobacterium tumefaciens*. The *ptl* operon is located downstream from the PT operon, is transcribed by the PT promoter, and codes for a type IV secretion system (Weiss *et al*., 1993; Covacci and Rappuoli, 1993; Ricci *et al*., 1996; Farizo *et al*., 2000). The *Ptl* secretion machinery mediates the translocation of the PT holotoxin across the outer membrane. However, recent data provide evidence that the S1 subunit localizes to the outer membrane prior to assembly with the B oligomer and independently from the *Ptl* system. Therefore, the membrane-bound S1 may serve as a nucleation site for assembly with the B oligomer and for subsequent interaction with *Ptl* proteins (Farizo *et al*, 2002).

The amino acid sequence of subunit S1 shows a significant homology with the A1 promoter of cholera and *E. coli* LT toxins (see Figure 12.10) (Nicosia *et al*.,

**TABLE 12.1** Toxic and non-toxic properties of PT and PT–9K/129G mutant

| Property | Native PT | PT–9K/129G mutant | Reference |
|---|---|---|---|
| **Toxic properties of PT** | | | |
| CHO cell-clustered growth (ng $ml^{-1}$) | 0.005 | >5000[a] | Pizza *et al*. (1989) |
| Histamine sensitization (μg $mouse^{-1}$) | 0.1–0.5 | >50[a] | Nencioni *et al*. (1990) |
| Leucocytosis stimulation (μg $mouse^{-1}$) | 0.02 | >50[a] | Nencioni *et al*. (1990) |
| Anaphylaxis potentiation (μg $mouse^{-1}$) | 0.04 | >7.5[a] | Nencioni *et al*. (1990) |
| Enhanced insulin secretion (μg $mouse^{-1}$) | <1 | >25[a] | Nencioni *et al*. (1990) |
| IgE induction (*in vitro*) (ng $ml^{-1}$) | 0.8 | >100[a] | van der Pouw-Kraan *et al*. (1995) |
| IgE induction (*in vivo*) (ng $rat^{-1}$) | 10 | >200[a] | van der Pouw-Kraan *et al*. (1995) |
| Long-lasting enhancement of nerve-mediated intestinal permeabilization of antigen uptake (ng $rat^{-1}$) | 1 | >200[a] | Kesecka *et al*. (1994) |
| Inhibition of IL-1-induced IL-2 release in ELA 6.1 cells (μg $ml^{-1}$) | 0.1 | >100[a] | Zumbihl *et al*. (1995) |
| Inhibition of neutrophil migration (μg $rat^{-1}$) | 0.2 | >1.2[a] | Brito *et al*. (1997) |
| Lethal dose (μg $kg^{-1}$) | 15 | >1500[a] | Unpublished data |
| ADP-ribosylation (ng) | 1 | >20 000[a] | Pizza *et al*. (1989) |
| **Non-toxic properties of PT** | | | |
| T-cell mitogenicity (μg $ml^{-1}$) | 0.1–0.3 | 0.1–0.3 | Nencioni *et al*. (1991) |
| Haemagglutination (μg $well^{-1}$) | 0.1–0.5 | 0.1–0.5 | Nencioni *et al*. (1991) |
| Mitogenicity for PT-specific T-cells (μg $ml^{-1}$) | 3 | 3 | Pizza *et al*. (1989) |
| Platelet activation (μg $ml^{-1}$) | 5 | 5 | Sindt *et al*. (1994) |
| Mucosal adjuvanticity (μg $mouse^{-1}$) | 3 | 3 | Roberts *et al*. (1995) |
| Affinity constant (monoclonal 1B7, anti-$S_1$) | $2.4 \times 10^8$ | $6.1 \times 10^8$ | Nencioni *et al*. (1990) |
| Affinity constant (polyclonal anti-PT) | $2.0 \times 10^{10}$ | $9.8 \times 10^9$ | Nencioni *et al*. (1991) |

[a]No effect was observed at the highest dose reported used in the assay.

1986; Locht and Keith, 1986). The crystal structure of the toxin (Figure 12.11) has revealed a structural similarity to the catalytic subunits of the other ADP-ribosylating toxins and a structure of the B subunit that resembles that of cholera toxin organized around a central cavity composed by the α-helices of each subunit (Stein *et al*., 1994b).

### *Mutants of pertussis toxin*

The role of many amino acids of the S1 subunit has been tested by site-directed mutagenesis initially performed to produce non-toxic mutants of PT to be used as vaccines. The minimal region still enzymatically active contains amino acids 4–179 (Pizza *et al*., 1988; Cieplak *et al*., 1988; Barbieri and Cortina, 1988). Within this region, many amino acids have been changed by site-directed mutagenesis (Figure 12.7; Barbieri and Cortina, 1988; Pizza *et al*., 1988; Burnette *et al*., 1988; Black *et al*., 1988; Kaslow *et al*., 1989; Locht *et al*., 1989; Lobet *et al*., 1989). Arg9, Asp11, Arg13, Trp26, His35, Phe50, Glu129, and Tyr 130 were found to be essential for enzymatic activity. In fact, their replacement with other amino acids reduced the activity of recombinant S1 molecules to levels equal or below 1%. Most of these amino acids could be replaced without altering the overall structure of the recombinant S1 molecule, which was still recognized by toxin-neutralizing monoclonal antibodies specific for a conformational epitope. In addition to the amino acids described previously, Cys41 was shown to be close to the active site since its alkylation decreased enzymatic activity (Kaslow *et al*., 1989). By photoaffinity labeling with NAD+, Glu129 was shown to be equivalent to Glu148 of DT and Glu553 of PAETA (Barbieri *et al*., 1989). When the above amino acid changes were introduced either alone or in combination into the PT operon in the *B. pertussis* chromosome, a number of mutant PT molecules were obtained; most of them had a polyacrylamide gel electrophoretic pattern indistinguishable from the wild-type PT and a reduced toxicity. Some mutants were

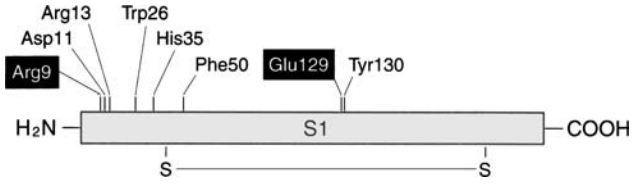



**FIGURE 12.7** Toxic subunit $S_1$ of the pertussis toxin (PT) and illustration of some of the residues that have been mutagenized. In particular, double substitution of Arg9 and Glu129 (black boxed) produces the most well-known mutant that has been used for the construction of an acellular vaccine against pertussis.

unable to assemble the S1 subunit and released into the extracellular medium only the B oligomer. These mutants contain mutations in any of the two cysteines, in regions homologous to cholera toxin (8D/9G, 50E, 88E/89S), or contain three amino acid changes as in mutant 13L/26I/129G. The above mutations are believed to alter the structure of the S1 subunit and prevent its assembly into the holotoxin (Pizza *et al*., 1990). In general, the molecules containing single amino acid mutations had a toxicity reduced from 4- to 1000-fold, but none of them was completely non-toxic. The mutant that has become most famous contains two amino acid substitutions: Arg 9 → Lys and Glu 129 → Gly (PT-9K/129G). This mutant has a structure indistinguishable from wild-type, but is completely free of any toxicity (see Table 12.1). It has been used for the construction of a vaccine against pertussis that has been extensively tested in clinical trials and shown to induce protection from disease. The vaccine containing this mutant is presently licensed in several countries (Pizza *et al*., 1989; Rappuoli 1997).

Mutants in the B oligomer have also been constructed. The most relevant is the one containing deletion of Asn105 in S2 and Lys105 in S3, which resulted in drastic reduciton of binding (Lobet *et al*., 1993).

*Cholera toxin and* Escherichia coli *enterotoxin*

Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) are AB toxins sharing high homology (95% identity) in their primary structure (Dallas and Falkow, 1980; Spicer *et al*., 1981) and super-imposable tertiary structures (Sixma, 1991). Both toxins are composed of a pentameric B oligomer that binds the receptor(s) on the surface of eukaryotic cells and an enzymatically active A subunit that is responsible for the toxicity (Holmgren, 1981; Moss and Vaughan, 1988) (see Figure 12.1). Both the A and B subunits of CT and LT are synthesized intracellularly as precursor proteins that, after removal of the leader peptide and translocation across the cytoplasmic membrane, assemble in the periplasmic space to form the final $AB_5$ complex. While *V. cholerae* exports the CT toxin into the culture medium, LT remains associated to the outer membrane bound to LPS (Horstman and Kuehn, 2002). The A subunit is composed of a globular structure linked to the B oligomer by a trypsin-sensitive loop and a long α-helix whose carboxyterminus enters into the central cavity of the B oligomer, thus anchoring the A subunit to the B pentamer (Sixma, 1991). Following protease cleavage of the loop, the A subunit is divided into the globular (enzymatically active) $A_1$ and the carboxy-terminal $A_2$ fragments that remain linked by a disulfide bridge between the $A_1$-cys187 and the $A_2$-cys199. Proteolytic cleavage of the loop and reduction of the disulfide bridge are both necessary in order to generate the enzymatic activity (Gill and Rappaport, 1979). The loop is intact when the molecules are produced in *E. coli*, while it is already cleaved when molecules are produced in *Vibrio cholerae*, which produces a specific protease to cleave this loop. The $A_1$ subunit contains the ADP-ribosylating activity and transfers the ADP-ribose group to the stimulatory α subunit of Gs. Once this G protein is ADP-ribosylated, the adenylate cyclase is permanently activated, causing abnormal intracellular accumulation of cAMP (Field *at al*., 1989a; Field *et al*., 1989b). While cAMP accumulation is believed to be responsible for the toxicity of CT and LT, we cannot exclude a contribution of interactions with less well-characterized G proteins to the toxicity.

The active site of the A subunit has a structure similar to all enzymes with mono-ADP-ribosylating activity (see Figure 12.11; Domenighini *et al*., 1994; Domenighini and Rappuoli, 1996). The catalytic site is formed by a cavity with a β-strand (the floor of the cavity) followed by an α-helix (the ceiling of the cavity) that contains two amino acids that are essential for catalysis: these are Arg7 (Burnette *et al*., 1991; Lobet *et al*., 1991) and Glu110–Glu112 (Tsuji *et al*., 1990; Tsuji *et al*., 1991). A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa guanine-nucleotide binding proteins, known as ADP-ribosylation factors or ARFs (Tsai *et al*., 1988).

ARF proteins are known not only to function as activators of CT and LT, but also to play a crucial role in vesicular membrane trafficking in both endocytic and exocytic pathways, maintenance of organelle integrity, and assembly of coat proteins in eukaryotic cells. The most abundant and the best known ARFs family members are class I ARF, which are involved in the endoplasmic reticulum (ER)-Golgi and intra-Golgi transport. Like all GTP-binding proteins, ARF can bind GDP or GTP. The GDP-bound form is present in the cytosol. Initiation of vesicles' formation occurs when a Golgi membrane enzyme interacts with the ARF-GDP complex and catalyzes the release of GDP and the binding of GTP. The ARF-GTP form mediates the assembly of cytosolic coat proteins (coatomers) to Golgi membranes, inducing budding of the transported vescicles, (Donaldson and Klausner, 1994; Moss and Vaughan, 1995; Boman and Kahn, 1995). Therefore, we can see the A1 fragment as having at least two independent functions: enzymatic activity and ARF binding. The enzymatic activity takes place within the eukaryotic cells and can be measured *in vitro*; whether ARF activates CT and LT in the eukaryotic cells remains unknown. While the region of LT and CT carrying the catalytic site is well-characterized, so far we have no

idea of where the ARF binding site is localized on the A1 subunit. However, it is known that both trypsinization and reduction of the A subunit are needed for expression of a functional ARF binding site (Moss *et al.*, 1993), and in a study on non-toxic derivatives of LT it has been shown that the two sites (catalytic and ARF binding) are independent and located on different regions of the A1 domain (Stevens *et al.*, 1999). The B oligomer is a pentameric molecule of 55 kDa, containing five identical polypeptide monomers. The structure is compact, resistant to trypsin, and requires boiling in the presence of sodium dodecyl sulfate to be dissociated. The five subunits are arranged in a cylinder-like structure with a central cavity that on one side exposes five symmetrical cavities that are responsible for binding to the eukaryotic cell receptor (Sixma *et al.*, 1991; Sixma *et al.*, 1993). The receptor binding site is specific for a variety of galactose-containing molecules and shows a different fine specificity between LT and CT. CT binds mostly to the ganglioside GM1 that is believed to be the major toxin receptor (Holmgren, 1973), while LT binds not only to GM1 but also to other glycosphingolipids (Teneberg *et al.*, 1994), to glycoprotein receptors present in the intestine of rabbits and humans (Holmgren *et al.*, 1982; Holmgren *et al.*, 1985; Griffiths *et al.*, 1986), to polyglycosilceramides (PCGs) (Karlsson *et al.*, 1996), and to paragloboside (Teneberg *et al.*, 1994). Furthermore, the two variants of LT, human LT (hLT) and porcine LT (pLT) that differ only by four amino acids (Domenighini *et al.*, 1995b), are identical in their binding to glycoproteins and PCGs, but different in binding to paragloboside. Only pLT and not hLT is able to bind paragloboside, and the reason seems to be due to the differences in the residue in position 13, which is an Arg in pLT and an His in hLT (Karlsson *et al.*, 1996). The different receptor-binding activities of the molecules may be significant for the qualitatively different immunological properties exhibited by LT and CT. The B oligomer can be produced in great quantities from *E. coli* transformed with the B subunit gene under the control of a strong promoter (Lebens *et al.*, 1993). The monomeric subunits are individually secreted into the periplasmic space where they are assembled into the pentameric structure (Hofstra and Witholt 1985). CTB and LTB are systemic and mucosal immunogens, and this property is dependent by the binding to the GM1 receptor. In addition, they are strong inhibitors of T cell activation and are able to induce apoptosis of $CD8^+$ T cells and, to a lesser extent, of $CD4^+$ T cells (Truitt *et al.*, 1998). On the basis of results of different studies, we have summarized in Figure 12.4 the sequence of events that take place during cell intoxication (Bastiaens *et al.*, 1996; Majoul *et al.*, 1996).

The holotoxin binds the receptor located on the eukaryotic cell membrane (Figure 12.4, stage 2). It is internalized into vesicles that, instead of taking the usual endocytic pathway leading to lysosomes, are transported retrogradally to the Golgi compartment (Figure 12.4, stages 2b and 2c). At this stage, the toxin is still in the $AB_5$ form. In the ER, the A1-chain of the CT unfolds and enters the cytosol by a process termed retro-translocation. Upon entering the cytosol, the A1-chain rapidly refolds, binds ARF (Figure 12.4, stage 2d) and induces toxicity by ADP-ribosylating the α subunit of $G_s$ located on the plasma membrane (Lencer and Tsai, 2003). The B subunits persist in the Golgi and are subsequently degraded.

***Mutants of cholera and Escherichia coli enterotoxin***

In order to study the structure-function of CT and LT and to find molecules that are non-toxic but still active as mucosal adjuvants and immunogens, more than 50 different site-directed mutants have been produced (Okamoto *et al.*, 1988; Tsuji *et al.*, 1990; Tsuji *et al.*, 1991; Burnette *et al.*, 1991; Lobet *et al.*, 1991;Lycke *et al.*, 1992; Pizza *et al.*, 1994a; Pizza *et al.*, 1994b; Fontana *et al.*, 1995; Douce *et al.*, 1995; de Haan *et al.*, 1996; Yamamoto *et al.*, 1997; Guidry *et al.*, 1998; Jobling and Holmes, 2001; O'Neal *et al.*, 2004). The most important ones are reported in Figure 12.4. Among these, the ones that have been best studied are LTK63, LTK97, and LTK7, for which the three-dimensional structure has been determined (Merritt *et al.*, 1995; van den Akker *et al.*, 1995; van den Akker *et al.*, 1997). One of the most interesting mutants is LTK97, where the Val → Lys mutation does not change at all the three-dimensional structure of the molecule but introduces a salt bridge between the charged amino group of Lysine 97 and the carboxylate of the glutamic acid 112, thus making it unavailable for further interactions. The observation that a simple hydrogen bond inactivates the enzymatic activity suggests an important role of the negative charge of the Glutamic acid in the enzymatic activity. Other interesting mutants are LTK63 and CTK63 (containing a serine-to-lysine substitution in position 63 of the A subunit). These holotoxoids have no detectable enzymatic activity and no toxicity *in vitro* or *in vivo*, even when huge amounts of them are used (Giannelli *et al.*, 1997; Giuliani *et al.*, 1998). Therefore, we can consider them as complete knock-outs of enzymatic activity but otherwise indistinguishable from wild-type. In fact, many other biological properties measured to date are maintained intact, including receptor and ARF-binding (Stevens *et al.*, 1998). The X-ray structure of LTK63 has shown complete identity to the wild-type LT across the entire molecule, with the exception of the active site, where the bulky side chain of Lys63 fills the catalytic

cavity, thus making it unsuitable for the enzymatic activity (see Figure 12.8, panel C) (van den Akker *et al.*, 1997). LTK63 is an excellent mucosal adjuvant although the activity is reproducibly reduced in comparison to LT (Partidos *et al.*, 1996; Di Tommaso *et al.*, 1996; Douce *et al.*, 1997; Ghiara *et al.*, 1997; Marchetti *et al.*, 1998; Giuliani *et al.*, 1998) while CTK63 is a less active mucosal adjuvant (Douce *et al.*, 1997; Pizza *et al.*, 2001).

A second class of mutant molecules contains LTR72 (containing an alanine-to-arginine substitution in position 72 of the A subunit) and CTS106 (containing a proline-to-serine substitution in position 106 of the A subunit). These mutants have approximately 1% of the wild-type ADP-ribosylating activity, have a toxicity *in vitro* in Y1 cells reduced by a factor of $10^4$–$10^5$ and a toxicity *in vivo* in the rabbit ileal loop reduced by

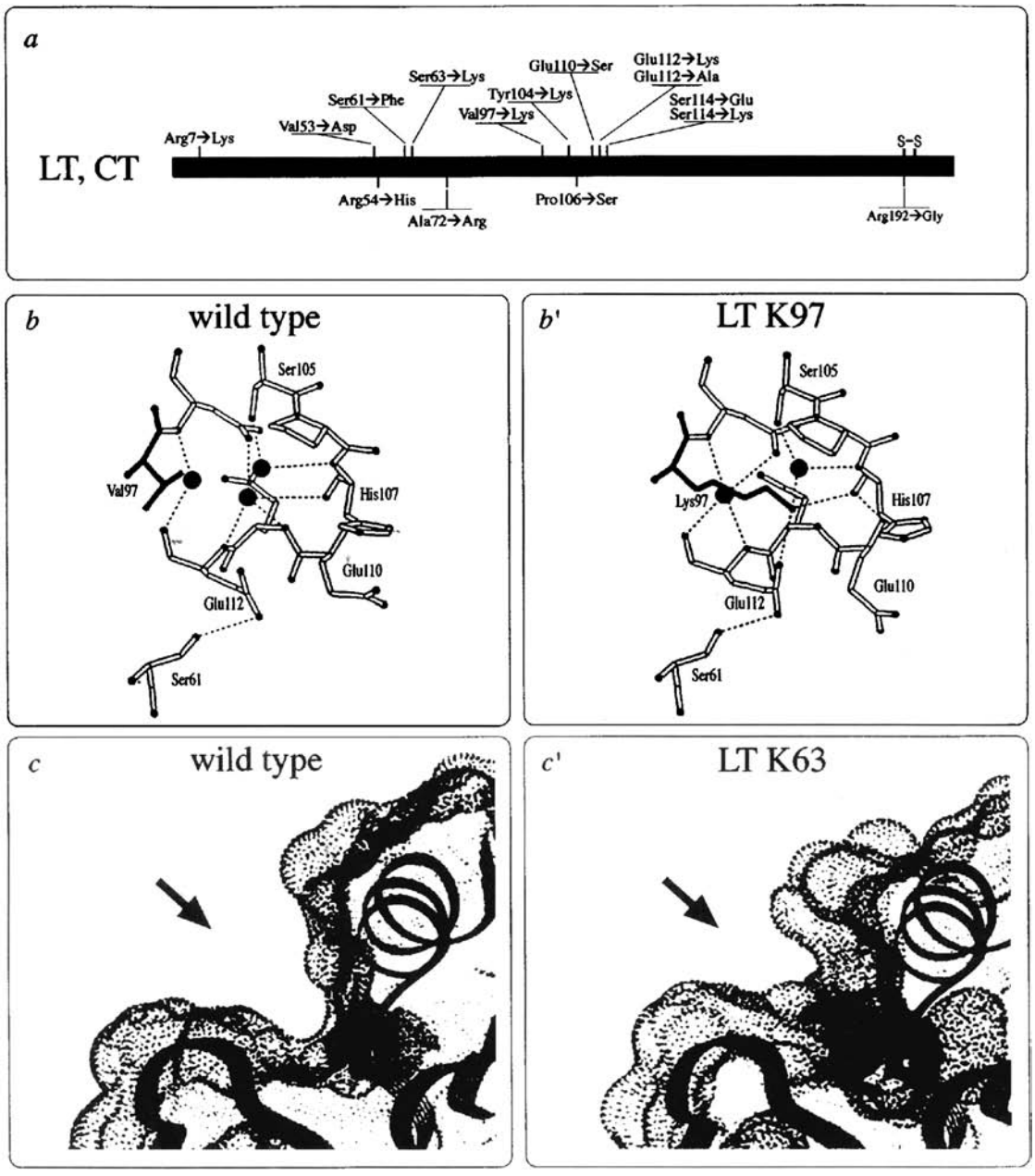



**FIGURE 12.8** (a) Schematic representation of LT–CT toxins, where the most relevant site-directed mutations are reported. Two of them are illustrated in more detail in (b) and (c). (b) Structure of the wild-type LT and of the mutant LT K97 (b′). Glu112 is involved in one hydrogen bond in the native structure and in two in the mutant. (c) Three-dimensional structure of the enzymic cavity of the wild-type LT and (c′) of the mutant LT K63. The arrows show how this single mutation can affect the dimensions of the pocket and thus the entrance of the NAD molecule.

25–100 fold (Figure 12.9). Both LTR72 and CTS106 are excellent mucosal adjuvants, being as effective as LT and CT, respectively (Douce *et al.*, 1997; Giuliani *et al.*, 1998; Pizza *et al.*, 2001).

Mutants in the protease sensitive loop have been also constructed with the aim of making the loop insensitive to proteases, and therefore the toxin not susceptible to the activation process that is necessary for enzymatic activity and toxicity. Among the many mutants constructed, LTG192 is the best characterized. In this LT mutant, the arginine in position 192 is replaced by a glycine (Grant *et al.*, 1994; Dickinson and Clements 1995; Giannelli *et al.*, 1997). *In vitro* the mutant is completely resistant to trypsin treatment. *In vivo*, proteases different than trypsin may partially cleave the loop and activate the toxin because toxicity is detectable. The toxicity observed in Y1 cells is approximately $10^3$ times lower than that of wild-type toxin during the first 8 hours of incubation and becomes only 5–10 times lower than wild-type following longer incubation (Giannelli *et al.*, 1997). In practice, this molecule takes longer to be activated but delivers approximately the same total enzymatic activity as wild-type. The difference is that the delivery of the active toxin is diluted over a longer period of time. *In vivo*, in the rabbit ileal loop, almost no difference in toxicity is observed between LTG192 and wild-type LT (Figure 12.9) (Giannelli *et al.*, 1997). Ongoing human trials are expected to establish the safety profile of these molecules (Vaccine Weekly, 1995).

Mutant derivatives of LTB have also been constructed. Those that are defective in receptor binding (for example LTB/D33, containing a glycine-to-aspartic acid substitution in position 33) were found to be almost completely non-immunogenic at mucosal surfaces, suggesting that an intact receptor-binding site is necessary both for binding and the immunogenicity associated with the molecule (Nashar *et al.*, 1996). Other studies with a non-receptor–binding mutant of LT (LTD33) have confirmed that receptor binding is necessary also for adjuvanticity (Guidry *et al.*, 1997). Interestingly, non-binding LTB mutants have also been shown to lose other immune-modulating activities, including their ability to induce apoptosis of CD4+ and CD8+ cells (Nashar *et al.*, 1996; Truitt *et al.*, 1998).

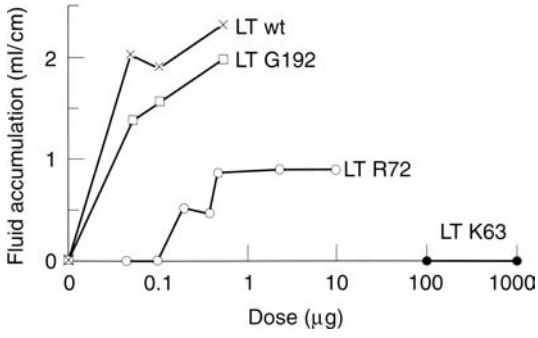



**FIGURE 12.9** Toxicity in the rabbit ileal loop of wild-type LT and of the most important mutants.

## Binary toxins and toxins that are directly delivered to the cytoplasm of eukaryotic cells

### Clostridium botulinum *C2 and iota-like toxins*

*C. botulinum* C2 toxin is a member of a family of "binary" cytotoxins that ADP-ribosylate monomeric G-actin at an Arginine in position 177 (Vandekerckhove *et al.*, 1988). Being the Arginine at contact site between actin monomers, the binding of the ADP-ribose makes actin unable to polymerize (Aktories *et al.*, 1986). *C. botulinum* toxin C2 is an extremely toxic agent, which induces hypotension, increase in intestinal secretion, vascular permeability, and hemorrhaging in the lungs. C2 is composed of two separate molecules, the 50 kDa enzymatically active toxin (C2I) and a receptor-binding component (C2II) of 75 kDa, (Aktories and Wagner 1992). The organization of C2 resembles closely that of the EF, LF, and PA of *Bacillus anthracis*. Like PA, C2II is activated by proteolytic cleavage and forms ring-shaped heptameric pores on the membrane of host cells by a pH-mediated process. Following C2II oligomerization and binding to the host cell receptor, the toxic moiety binds to the heptameric pore and is translocated across the membrane (Blocker *et al*, 2003). Toxins related to C2 are *C. perfringens* iota toxin (Perelle *et al*., 1995), the mosquitocidal toxin (MTX) produced by *Bacillus sphaericus* (Schirmer *et al*., 2002), the *C. spiriforme* toxin (Stiles and Wilkins, 1986), the ADP-ribosyltransferase (CDT) produced by *C. difficile* (Popoff *et al*., 1988; Gulke *et al*., 2001), and the vegetative insecticidal protein VIP2 of *Bacillus cereus* (Han *et al*., 1999). The level of primary sequence homology detected among the enzymatic and binding components of this class of ADP-ribosylating toxin ranges from 32 to 80% identity, the binding domains being the better conserved.

The iota toxin is a binary toxin produced by *Clostridium perfringens* type E, which has been implicated in fatal calf, lamb, and guinea pig enterotoxemias. Structurally, it has two independent domains: Ia, which is the ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin by the cell (Marvaud *et al*., 2001). The crystallization of the C2-I component in complex with its substrate NADH has recently been achieved (Tsuge *et al*., 2003), showing a close relationship of iota toxin with insecticidal protein VIP2 of *Bacillus cereus*, whose structure is also available (Han *et al*., 1999).

*Pseudomonas aeruginosa ExoenzymeS and related proteins*

Some toxins do not have a receptor-binding component and a translocation domain and are directly injected by bacteria into the cytoplasm of eukaryotic cells. In this case, bacteria intoxicate individual eukaryotic cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells. ExoS of *P. aeruginosa* and its homologue ExoT have been shown to reach the cytoplasm of eukaryotic cells by delivery through a specialized type III secretion system (Frithz-Lindsten *et al.*, 1997; Sundin *et al.*, 2004). For convenience, we also include here the C3 toxin of *Clostridium botulinum* for which a receptor-binding and translocation domain is not known.

The exoenzyme S is a 49 kDa bifunctional type-III cytotoxin of *Pseudomonas aeruginosa*. The N-terminal 233 residues comprise the Rho GTPase-activating protein (Rho GAP) domain, while residues 234–453 comprise the ADP-ribosyltransferase domain. The GTPase activating domain (GAP) of ExoS has been crystallized (Wurtele *et al.*, 2001). ExoS ADP-ribosylates the small G protein Ras at position 41 (Ganesan *et al.*, 1998). In order to become enzymatically active, ExoS requires the interaction with a cytoplasmic factor named FAS or 14.3.3 (Coburn *et al.*, 1991). The toxin is injected into eukaryotic cells by a type III secretion system (Frithz-Lindsten *et al.*, 1997). When cells are transfected with the *exoS* gene under the control of an eukaryotic cell promoter, collapse of the cytoskeleton and a change of the cell morphology that results in the rounding up of the cells are observed. Recent data suggest that *in vivo*, ExoS may contribute to the establishment of *P. aeruginosa* infections by inhibiting wound healing and tissue regeneration by ADP-ribosylating Ras and therefore inhibiting the cellular motility and angiogenesis required for wound healing. Through the inhibition of tissue regeneration and wound healing, ExoS may play a pivotal role in chronic disease by maintaining sites of colonization (Barbieri, 2000).

ExoT is 75% homologous to ExoS; however, when compared with ExoS, it exhibits highly reduced ADP-ribosylating activity, but still exhibits GAP (GTPase activating protein) activity on RhoA GTPase *in vitro* (Sundin *et al.*, 2001). More recent data have suggested that ExoT ADP-ribosylates Crk-I and Crk-II (CT10-regulator of kinase) that play an essential role in integrin-mediated phagocytosis and focal adhesion (Sun and Barbieri, 2003). ADP-ribosyltransferase activity of ExoT mediates changes in cell morphology, which correlate to disruption of the actin microfilaments of the infected cells (Sundin *et al.*, 2004).

Finally, another protein belonging to the group of ExoS-related toxins is exoenzyme T (AexT) of *Aeromonas salmonicida*, a fish pathogen implicated in furunculosis. The amino acid sequence of AexT closely resembles those of ExoS and ExoT, and antibodies directed against AexT cross-react with ExoS and ExoT. Expression of AexT occurs specifically during infection of the host and causes cell damage, suggesting that this novel ADP-ribosyltransferase is a key virulence determinant of *A. salmonicida* (Braun *et al.*, 2002). Similarly to *P. aeruginosa*, *A. salmonicida* also encodes a type III secretion system, which is utilized for AexT export across the membrane and into the cytosol of eukaryotic cells (Burr *et al.*, 2003).

Clostridium botulinum *exoenzyme C3 and related proteins*

Exoenzyme C3 is a protein of 211 amino acids that is produced by *C. botulinum* (Aktories *et al.*, 1989; Chardin *et al.*, 1989) and that *in vitro* ADP-ribosylates the small regulatory protein Rho at Asparagine 41 (Sekine *et al.*, 1989), thus inactivating its function. If the protein is microinjected into cells or the cells are transfected with the C3 gene under an eukaryotic promoter, this causes disruption of actin stress fibers, rounding of the cells, and formation of arborescent protrusions (Hill *et al.*, 1995). However, for the moment, we do not know whether C3 alone is able to enter cells and intoxicate them because no mechanism of cell entry has been found.

Other members of this family of C3-related exoenzymes have been isolated from Gram-positive bacteria, such as certain strains of *Staphylococcus aureus* (Sugai *et al.*, 1992), *Clostridium limosum* (Just *et al.*, 1992), and *Bacillus cereus* (Just *et al.*, 1995). Whereas *C. botulinum* C3 and *C. limosum* exoenzyme are about 70% homologous and immunologically related, the epidermal cell differentiation inhibitor (EDIN) produced by *S. aureus* is only 35% homologous with C3 and shows no immunological cross-reactivity. However, crystal data recently obtained for *S. aureus* C3 exotoxin (EDIN-B) have disclosed a very similar structure (Evans *et al.*, 2003). *Bacillus cereus* exoenzyme exhibits the same substrate specificity as the other C3-like transferases; nevertheless, some differences can be observed for this toxin, such as the higher molecular weight (28 kDa) and, more importantly, the lack of immunological relationship to any other member of this family (Just *et al.*, 1995).

## Novel bacterial ADP-ribosylating toxins detected by genome-mining

With the advent of the Genomic Era, identification of bacterial factors possibly involved in virulence can be easily approached using computer-based techniques. In fact, given the vast amount of information that we now possess on toxins—including sequence data—and thanks to the growing number of sequenced bacterial

genomes, it is possible to proceed by homology criteria in order to predict novel members of important classes of bacterial toxins. Several examples exist where computer-based methodologies have been instrumental to the identification of novel potential bacterial toxins in sequenced genomes.

In the specific case of mono-ADP-ribosyltransferases (mADPRTs), these proteins display limited overall conservation at the primary structure level; however, the catalytic subunits show remarkable similarity within the enzymatic cavity where they are significantly well-conserved.

Given their importance in the virulence of related microorganisms, a series of studies have been directed towards the computer-based identification of novel ADP-ribosylating toxins by sequence-homology criteria in finished and unfinished genomes.

One of these works used sequence information and a database search to predict the ADP-ribosyltransferase activity of SpvB, a previously known virulence factor produced by *Salmonella* strains (Otto *et al*., 2001). Sequence analysis revealed that SpvB is composed of two domains, of which the C-terminus contains features characteristic of ADP-ribosylating factors like VIP2 and iota toxin. SpvB uses actin as a substrate and depolimerizes actin filaments when expressed in CHO cells. Its cytotoxicity is required for intracellular proliferation of *Salmonella* and represents the first ADP-ribosylating enzyme acting as a virulence factor for an intracellular pathogen.

In a subsequent paper, Pallen and colleagues (Pallen *et al*., 2001) reported the use of specialized homology search methods for the identification of novel putative ADP-ribosyltransferases encoded by diverse Gram-positive and Gram-negative bacterial species. All these predicted ADPRT belong to the group of CT-like enzymes and lack a predicted translocation domain (Table 12.2). Despite the absence of experimental data for most of them, sequence data indicate a possible role of these proteins in the pathogenesis of the corresponding microorganisms.

So far, only one of these predicted toxins, produced by *Streptococcus pyogenes* (SpyA), has been investigated to prove its ADP-ribosyltransferase activity and to assess its capability of entering eukaryotic cells.

SpyA is a protein of 250 amino acids, which shares good sequence homology to *C. botulinum* C3 exotoxin and to *S. aureus* EDIN. The recombinant protein has NAD-glycohydrolase and ADP-ribosyltransferase activities *in vitro* and modifies Arginine residues. *In vivo*, SpyA modifies numerous cytoskeletal proteins, such as vimentin, tropomyosin, and actin, therefore suggesting its direct involvement in cytoskeleton disruption and host colonization (Coye and Collins, 2004).

Very recently, a new protein has been added to the list of ADP-ribosyltransferases detected by computer analysis (Masignani *et al*., 2003). This novel factor has been identified by means of primary and secondary structure analysis in the genomic sequence of a virulent isolate of *Neisseria meningitidis* and has been named NarE (Neisseria ADP-ribosylating enzyme). As predicted by "in silico" studies, biochemical analysis has demonstrated that NarE is capable of transferring an ADP-ribose moiety to a synthetic substrate.

**TABLE 12.2** New ADP-ribosyltransferases detected by genome analysis

| Species | ADPRT | Length (aa) | Leader peptide | Features | Reference |
|---|---|---|---|---|---|
| *Bacillus halodurans* | BH3531 | 490 | no | Similar to phage structures | Pallen *et al.*, 2001 |
| *Burkholderia cepacia* | ART1-ART5 | 886–1283 | no | Detected on unfinished genome | Pallen *et al.*, 2001 |
| *Clostridium acetobutylicum* | ART_CLOAB | 388 | no | ART domain at the C-terminus | Pallen *et al.*, 2001 |
| *Enterococcus faecalis* | ART1_ENTFA | 487 | no | Similar to phage protein | Pallen *et al.*, 2001 |
| | ART2_ENTFA | 423 | no | Similar to phage protein | Pallen *et al.*, 2001 |
| *Mycobacterium avium* | ART_MYCAV | 180 | no | ART domain only | Pallen *et al.*, 2001 |
| *Mycoplasma pneumoniae* | YD72_MYCPN | 591 | yes | Similar to PT S1 | Pallen *et al.*, 2001 |
| *Pseudomonas syringae* | ART1-ART5 | 182–300 | no | ART domain mostly | Pallen *et al.*, 2001 |
| *Salmonella typhi* | TOXA_TYPHi | 242 | yes | Similar to PT S1 | Pallen *et al.*, 2001 |
| *Streptomyces coelicolor* | SCO5461 | 219 | yes | Secreted protein | Pallen *et al.*, 2001 |
| *Vibrio parahemolyticus* | TH3996 | 275 | no | none | Pallen *et al.*, 2001 |
| *Salmonella spp* | SpvB | 591 | yes | ADP-ribosylating activity shown | Otto *et al.*, 2001 |
| *Streptococcus pyogenes* | SpyA | 250 | yes | Similar to EDIN, ADP-ribosylating activity shown | Coye and Collins, 2004 |
| *Neisseria meningitidis B* | NarE | 145 | no | Secreted in the periplasm, ADP-ribosylating activity shown | Masignani *et al.*, 2003 |

## EUKARYOTIC MONO-ADP-RIBOSYLTRANSFERASES

Eukaryotic mono-ADP-ribosyltransferases (ARTs) are ectoenzymes produced by vertebrates, but absent in lower eukaryotes, like yeast, worms, flies, and mustard weed. Recent database searches and structure prediction methodologies have confirmed the presence of a limited number of ARTs in human and mouse genomes (Glowacki *et al*., 2002). Despite their limited primary sequence homology, their deduced amino acid sequences have similarities to those of viral and bacterial toxin enzymes in the region of the active site cleft, which is consistent with a common mechanism of NAD-binding and ADP-ribose transfer (Domenighini and Rappuolis 1996). Common substrate recognition domains have not yet been identified (Han and Tainer, 2002).

In vertebrates, this posttranslational protein modification can be used to control important endogenous physiological functions, such as the induction of long-term potentiation in the brain, terminal muscle cell differentiation, and the cytotoxic activity of killer T cells (McMahon *et al.*, 1993; Zolkiewska and Moss, 1993; Schumann *et al.*, 1994; Wang *et al.*, 1994). More recently, since ARTs are expressed preferentially in cells of the immune system, their possible involvement in the immune response has also been proposed (Paone *et al*., 2002).

The first vertebrate ribosyltransferases were purified and sequenced from chicken bone marrow and from rabbit and human skeletal muscle, and their specific target proteins have been identified. The majority of eukaryotic enzymes are arginine-specific transferases; nonetheless, ADP-ribosylation of cysteines was reported in bovine and human erythrocytes and platelet membranes (Saxty and van Heyningen, 1995; Tanuma *et al.*, 1988).

The family of mammalian enzymes consists of five proteins (ART1–5), which share extensive similarities in their gene structure and amino acid sequence (Okazaki and Moss, 1998). ART1–ART4 are all glycosylphosphatidylinositol (GPI)-anchored membrane proteins, with an extracellular catalytic domain, whereas ART5 possesses an N-terminal signal sequence that directs its secretion.

The rabbit ART1 is a 36kDa protein and its deduced amino acid sequence possesses hydrophobic amino- and carboxy-terminal signal peptides that are characteristic of GPI-linked proteins. There is roughly 75% of sequence identity among ART1 muscle enzymes isolated from humans, rats, and rabbits, this feature being consistent with considerable conservation across species. Like CT and LT, the muscle transferases specifically use the guanidino group of arginine as an ADP-ribose acceptor. The α7 integrin has been shown to be the target protein for cell surface mono-ADP-ribosylation in muscle cells. The ART1 enzymes have significant amino acid sequence identity to the RT6 (ART2) family of rodent T cell differentiation and activation antigens (Takada *et al.*, 1995). Rat and mouse ART2 sequences are roughly 80% identical, while in humans and chimpanzees, the ART2 genes contain three premature stop codons and thus appear not to be expressed. Recent studies suggest that ART2 may trigger T cell apoptosis (Liu *et al*., 2001). ART3 and ART4 were cloned from human testis and spleen, respectively, and they possess several regions of sequence similarity with ART1.

An ART5 cDNA was cloned from Yac-1 murine lymphoma cells, and its deduced amino acid sequence has similarities to other ART proteins in regions believed to be involved in catalytic activity.

Other mammalian ADP-ribosyltransferases have been purified from rat brain and adrenal medulla (Fujita *et al.*, 1995). These enzymes have been shown to modify, to different degrees, β/γ-actin, smooth muscle γ-actin, $G_s$, $G_i$, and $G_0$. The *in vitro* modification of brain and adrenal G proteins suggests potential mechanisms for cell signaling, similar to those observed with the bacterial toxins.

Previous studies have shown that the bacterial ADP-ribosyltransferases share three separate regions of similarity in their amino acid sequences, which seem to be also present in vertebrate ART enzymes. Alignment of these domains highlights conserved residues within the catalytic sites of the mammalian and bacterial toxin ADP-ribosyltransferases (Okazaki and Moss, 1994; Figure 12.11).

Recently, the three-dimensional structure of ART2 from rats has been solved (Mueller-Dieckmann *et al*., 2002). Detailed analysis reveals that, apart from the catalytic cleft, which is maintained among all ADP-ribosyltransferases, the global fold of ART2 is more closely related to VIP2 and C3 exoenzymes than to other bacterial ADP-ribosylating toxins.

Moreover, the deduced amino acid sequences of human poly(ADP-ribose) polymerases (PARPs) and perhaps ART3 appear to have regions of similarity that align with DT and PAETA; to support this observation, crystal structure of the chicken PARP (Ruf *et al.*, 1996) and mutagenesis of human PARP (Marsischky *et al.*, 1995) demonstrated that Glu 988, which is essential for ADP-ribose chain elongation, is positioned in a cleft similar to that found in bacterial toxins.

These data are consistent with the hypothesis that several of the bacterial toxins and vertebrate transferases possess a common mechanism of NAD binding and ADP-ribose transfer, and that differences observed

in the three-dimensional structures may reflect differences in substrate proteins.

## A COMMON STRUCTURE OF THE CATALYTIC SITE

Enzymes with ADP-ribosylating activity include toxins organized in an AB structure such as PAETA, CT, LT, toxins directly injected into eukaryotic cells such as ExoS and ExoT, phage-encoded proteins, and the ART family of proteins found in eukaryotic cells.

The analysis of the sequences of ADP-ribosylating enzymes known to date shows that all enzymes can be divided into two groups (Figure 12.10): the DT-like group, mainly composed of DT, PAETA, and human poly-ADP-ribosylpolymerases (PARPs), and the CT-like group comprising CT, LT, PT, C2, and C3 exoenzymes, MTX, ExoS, other bacterial ADP-ribosyltransferases, and eukaryotic ART enzymes. The obvious question is whether all these enzymes that perform the same reaction on different substrates have something in common.

While some homology is present within the CT family, overall no significant and extended sequence similarity can be detected to justify the observed common mechanism of catalysis. Nevertheless, biochemical experiments of photoaffinity labeling and studies of site-directed mutagenesis had previously demonstrated that all the toxins possessed a catalytic glutamic acid so important for enzymatic activity that not even a conservative substitution with an aspartic acid could be tolerated without loss or drastic decrease of toxicity. The structural identity of the active site was later confirmed by the crystal structure of several enzymes and computer modeling studies. This is formed by a β-strand followed by a slanted α-helix that has a different length in the various toxins. The β-strand and the α-helix represent, respectively, the lower and upper face of the cavity in which the nicotinamide ring of NAD enters and is anchored during the enzymatic reaction (Figure 12.11). The three-dimensional structure recently obtained for the *C. botulinum* C3 exoenzyme has shown that this toxin, although preserving the ADP-ribosyltransferase activity, lacks the elongated alpha helix, which generally constitutes the ceiling of the active site cleft in the ADP-ribosylating toxins crystallized so far. Seemingly, this feature does not impair the ability of C3 either to accommodate the NAD substrate or to carry out the enzymatic reaction (Han *et al*., 2001).

| Toxin | region 1 | region 2 | region 3 |
|---|---|---|---|
| **CT-like group** | ⇨ | ∿⇨ooooooooo | ⇨ |
| Pertussis toxin | VYRY | FV**ST**S**S**SRRYTEVYLEHRMQE | YQS**E**YL |
| Cholera toxin | IYRA | GYV**ST**S**I**SLRSAHLVGQTI | DEQ**E**VSAL |
| E. coli Heat-labile enterotoxin | IYRA | GYV**ST**S**L**SLRSAHLAGQSI | YEQ**E**VSAL |
| P. aeruginosa Exoenzyme S | – | GYL**ST**S**L**NPGVARSGQGTI | NEK**E**ILY |
| P. aeruginosa Exoenzyme T | – | GYL**ST**S**R**DPGVARSFAGQG | DEK**E**ILY |
| Aeromonas salmonicida AexT | – | AYL**ST**S**R**DPKVATNFG | DEQ**E**ILYN |
| C. botulinum C2 | AYRR | SF**ST**S**L**KSTPLSFS | DEQ**E**ILLN |
| C. perfringens Iota toxin | VYRR | FI**ST**S**I**GSVNMSAFAKRKI | GEY**E**VLLN |
| B. cereus VIP2 | VYRW | GYM**ST**S**L**SSERLAAFGSR | SEK**E**ILLD |
| B. sphaericus MTX | ILRW | FV**ST**T**R**ARYNNLGLEIT | NED**E**ITFP |
| C. botulinum C3 | LFRG | GYI**ST**S**L**MSAQFGGR | GQL**E**VLLP |
| C. limosum C3 | LFRG | GYI**ST**S**L**VNGSAFAGR | GQL**E**VLLP |
| S. aureus C3 (EDIN) | VYRL | GYS**ST**Q**L**VSGAAVGGR | GQQ**E**VLLP |
| Salmonella SpvB | YLRG | AFM**ST**S**P**DKAWINDT | GEA**E**MLFP |
| S. pyogenes SpyA | VYRY | SFM**ST**T**A**LKNGAMTHRP | SEV**E**LLFP |
| ART-1 mouse | VYRG | GFA**SA**S**L**KNVAAQQFGE | PEE**E**VLIPP |
| ART-2 rat | VYRG | FT**SSS**L**S**KKVAQSQ | DQE**E**VLIPG |
| ART-3 human | VYRT | – | KES**E**RITLIP |
| ART-4 mouse | VYHG | FL**SA**S**L**LKEE | LRK**E**VLIPP |
| ART-5 mouse | VFRG | FT**SSS**VDERVARR | EER**E**VLIPP |
| **DT-like group** | | | |
| Diphtheria toxin | SY**H**GT | WKGF**Y**STDNKYDAAG**Y** | **E**YIN |
| Pseudomonas exotoxin A | GY**H**GT | WRGF**Y**IAGDPALAYG**Y** | **E**TIL |
| PARP (consensus) | LW**H**GS | KGI**Y**FADMVSKSAN**Y** | **E**YIV |

**FIGURE 12.10** Sequence alignment of protein segments containing Regions 1, 2, and 3 of known ADP-ribosylating enzymes. The two groups of homology (DT-like and CT-like groups) are distinguished. Catalytic residues of Regions 1 and 3 and most relevant and conserved residues of Region 2 are in bold, and extended consensus sequences detected in the three regions are shaded. Predicted and observed secondary structure folding is indicated for each region: Region 1 and 3 are β-strands (arrows), while Region 2 is characterized by a short coil, followed by a β-strand and by a α-helix. PARP, human poly-ADP-ribosylpolymerases.

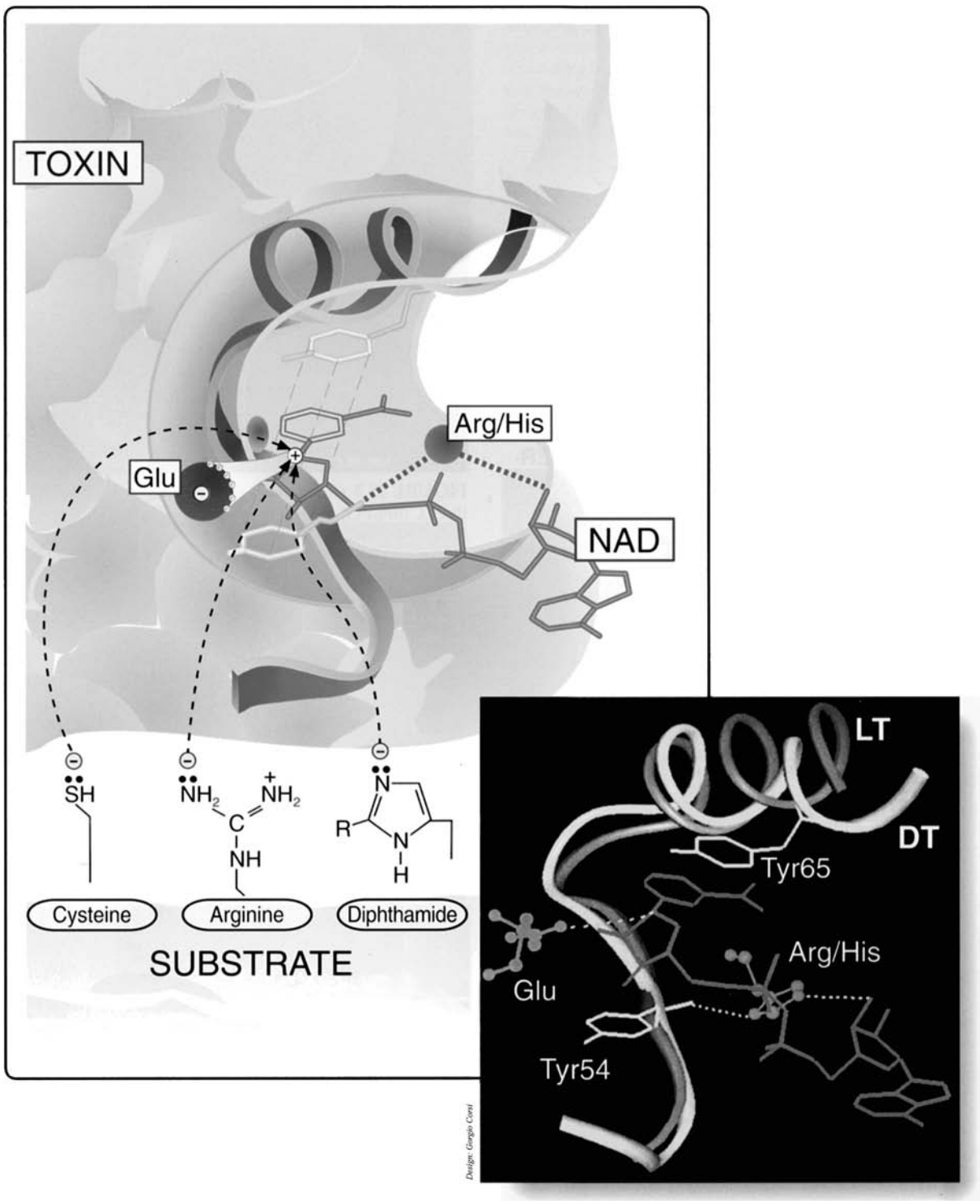



**FIGURE 12.11** Catalytic site of ADP-ribosylating toxins. Upper panel: a schematic representation of a possible common mechanism of catalysis is illustrated: the NAD molecule is docked inside the cavity by means of stacking interactions provided by the two aromatic rings that protrude from the scaffold of the $\beta$–$\alpha$ structure. The catalytic glutamic acid and its possible interactions with the acceptor residues of the various substrates are also reported. The Arg–His residue provides stabilizing interactions with the backbone of the cavity and seems also to be responsible for the correct positioning of NAD inside the pocket. Lower panel: superimposition of the backbones of the three-dimensional structures of the $\beta$–$\alpha$ motif of DT and LT. The NAD molecule has been introduced inside the cavity as it is folded in the complex with the DT structure (Bell and Eisenberg, 1997). The essential amino acids together with their main interactions are again reported.

Although all the toxins share a similar folding in the region of the catalytic domain, at the amino acidic level, the only residues that appear to be conserved among all representatives of the DT and CT subgroups are a glutamic acid and an histidine or an arginine; X-ray data have shown that they are carried by two independent β-strands and retain an equivalent spatial position and orientation in all the enzymes. The knowledge of the structure of the active site and of the amino acids that form it allowed us to go back to the primary sequences and find similarities that had been previously disregarded.

Briefly, all the mentioned toxins share three common features, namely the cavity formed by the β-α structure and the two catalytic residues (identified by numbers 2, 1, and 3 respectively in Figure 12.10). A closer look at these regions shows that the β-α structure (region 2) has very little or no amino acid sequence homology; however, it is identified by an ...S-X-S... motif in most of the enzymes of the CT group and by an ...Y-$(X)_{10}$-Y... in the DT group. The glutamic acid shown in Figure 12.10, (region 3) (namely Glu 148 of DT, Glu 553 of PAETA, Glu 112 of CT and LT, Glu 129 of PT, Glu 214 of C3 exoenzyme, etc.) is characterized by a Glu/Gln-X-Glu in the CT group. The other conserved amino acid (region 1) is an Arg in the CT group and a His in the DT group (His 21 of DT, His 440 of PAETA, Arg 7 of CT, Arg 9 of PT, Arg91 in C3, etc.). This region is usually identified by the Aro-Arg/His motif (region 1). The essential role of the amino acids in regions 1 and 3 and of the structure of the cavity in region 2 has been confirmed experimentally by site-directed mutagenesis. In the case of DT, it has been established that the carboxylate group of the side chain of Glu 148 lies near the nicotinamide ring of NAD, at a distance of 4 Å from the N-glycosidic bond. Extending this observation to all the toxins, the more likely explanation in terms of enzymatic activity is that the essential glutamic acid could play a role in stabilizing a chemical intermediate of NAD by forming a hydrogen bond with the O2′ hydroxyl group of the nicotinamide ribose (Han *et al.*, 1999). Although several models have been proposed to explain the possible function of the conserved histidine/arginine, it seems now widely accepted that this residue does not play a direct role in catalysis; very likely, it has the function of maintaining the integrity of the active-site pocket upon formation of structurally stabilizing hydrogen bonds.

The other reported block of homology is the domain β-α, which includes a number of amino acids that, while maintaining the same secondary structure in both DT and CT families, still strongly differ in terms of sequence. This region corresponds to the core of the active site cleft, which is devoted to the docking of NAD. The difference detected in terms of primary structure between the two subgroups suggests that the toxins may adopt a diverse mechanism of binding. The consensus sequence generated for the DT group is characterized by two conserved tyrosines spaced by 10 amino acids, and respectively located on the middle portion of the β-strand and on the internal face of the α-helix. Tyr 54 and Tyr 65 of DT, and Tyr 470 and Tyr 481 of PAETA have been shown to play a very important role in catalysis, since they anchor the nicotinamide ring creating a π pile of three aromatic rings that strengthen the overall binding of NAD and stabilize the complex. In PT, a similar role is likely to be played by Tyr 59 and Tyr 63, which have a similar spatial orientation and distance from each other. This observation is supported by the fact that in CT and LT, where the stacking interactions produced by the two tyrosines are lacking, the affinity for NAD is 1000-fold lower. In the case of the CT group, the box β-α is centered on a conserved core region characterized by the consensus Ser-Thr-Ser that is observed and predicted to fold in a β-strand that represents the floor of the cavity. No conclusive information is available to define the precise role of these small, polar residues in catalysis, but experiments of site-directed mutagenesis confirm that they are extremely important in maintaining the cavity available for NAD entrance and docking. Substitutions of Ser 61 and Ser 63 of LT with Phe and Lys, respectively, have been shown to produce non-toxic mutants.

Another amino acid that has been proposed as being important in catalysis is His 35 of PT, which is located near the beginning of the β-strand that forms the floor of the cavity in a position equivalent to that of His 44 of LT and CT; a functional homologue His is also present in MTX, but absent in DT and PAETA.

In the three-dimensional structure, this residue appears to be sufficiently close to the oxygen atom of the ribose ring of NAD to interact with it and increase the electrophilicity of the adjacent anomeric carbon atom. The absence of an equivalent residue in DT and PAETA again supports the idea that the two groups of toxins perform the same enzymatic activity in a slightly different fashion.

An additional feature that is common to all ADP-ribosylating toxins is the need for a conformational rearrangement in order to achieve enzymatic activity. In the native structure, the NAD binding site of LT and CT is obstructed by a loop comprising amino acids 47–56, which needs to be displaced in order to obtain a functional NAD-binding cavity. A functionally homologous region is also present in PT where the loop comprises residues 199–207. In the case of DT, where the crystallographic data of the complex are available, the observation

that the active site loop consisting of amino acids 39–46 changes structure upon NAD binding suggests that these residues may be important for the recognition of the ADP-ribose acceptor substrate, EF-2.

This proposal is supported by at least two lines of evidence: first, DT and PAETA have a high degree of sequence similarity in this loop region with a number of identical or highly conservative substitutions supporting the idea that these residues have some essential function; second, antibodies raised against a peptide corresponding to this loop sequence were able to block the catalytic domain of DT from catalyzing the ADP-ribosylation of EF-2.

The crystallographic data of the DT-NAD complex and the presence of common features within all ADP-ribosylating toxins allow us to speculate on a possible common mechanism of catalysis (Bell and Eisenberg, 1997).

The best hypothesis is that NAD enters the cavity upon displacement of the mobile loop, which is then made available for the recognition of the substrate; NAD is subsequently docked at the bottom of the pocket where a small residue (the conserved serine in the β-α box of the CT-group, the threonine 56 of DT, and the alanine 472 of PAETA) is required to allow good positioning. The nicotinamide moiety of NAD is then blocked in a suitable position by means of stacking interactions provided by a couple of aromatic rings (Tyr 54 and Tyr 65 of DT, Tyr 470 and Tyr 481 of PAETA, and possibly Tyr 59 and Tyr 63 of PT, respectively). In this context, the conserved arginine/histidine might display its key role in maintaining the correct shape of the active site pocket via hydrogen bonds formed with the backbone of the structure and possibly with one of the ribose moiety.

The enzymatic reaction is then catalyzed by the essential glutamic acid, which is likely to stabilize a positively charged oxocarbonium intermediate of NAD, in order to favor its subsequent interaction with the nucleophilic residue of the incoming substrate (Figure 12.11).

While a possible mechanism of reaction has been proposed, so far the residues implicated in substrate recognition have not been clearly mapped for any of the known ADPRTs. However, sequence alignment highlights the fact that all known Arg-specific ADP-ribosyltransferases contain a glutamic acid two residues upstream of the catalytic Glu (Figure 12.10). Mutation of this residue to glutamine has been shown to convert arginine-specific ADPRT activity into NAD-glycohydrolase activity in case of *C. botulinum* C2 toxin (Barth *et al*., 1998), *C. perfringens* iota toxin (Radke *et al*., 1999), and *Pseudomonas* exoenzyme S (Nagahama *et al*., 2000). On the other hand, all Rho-specific C3-like toxins display a Gln residue in this position, thus implying that this Gln may be required for Rho substrate recognition (Han *et al*., 2001).

Structural and mutagenesis studies of the NAD binding core of a binary toxin have identified an ARTT motif (ADP-ribosylating turn-turn motif) that is implicated in substrate specificity and recognition, and represents a general recognition region for most ADP-ribosylating enzymes (Han and Tainer, 2002). However, the ARTT motif seems to be only one of the several factors that determine substrate specificity. Very recently, in a study based on ExoS and ExoT in which the residues involved in substrate recognition were mapped, the authors proposed a model in which the substrate specificity is determined by the electrostatic potential in the enzyme-substrate interface (Sun *et al*., 2004).

## REFERENCES


Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K.H. and Habermann, E. (1986). Botulinum C2 toxin ADP-ribosylates actin. *Nature* **322**, 390–392.

Aktories, K., Braun, U., Rösener, S., Just, I. and Hall, A. (1989). The *rho* gene product expressed in *E.coli* is a substrate of botulinum ADP-ribosyltransferase C3. *Biochem. Biophys. Res. Comm*. **158**, 209–213.

Aktories, K. and Wegner, A. (1992). Mechanisms of the cytopathic action of actin-ADP-ribosylating toxins. *Mol. Microbiol*. **6**, 2905–2908.

Allured, V.S., Collier, R.J., Carroll, S.F. and McKay, D.B. (1986). Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Angstrom resolution. *Proc. Natl. Acad. Sc.i USA* **83**, 1320–1324.

Althaus, F.R. and Richter, C. (1987). ADP-ribosylation of proteins. Enzymology and biological significance. *Mol. Biol. Biochem. Biophys*. **37**, 1–237.

Anderson, P., Pichichero, M., Edwards, K., Porch, C.R. and Insel, R. (1987). Priming and induction of *Haemophilus influenzae* type b capsular antibodies in early infancy by Dpo20, an oligosaccharide-protein conjugate vaccine. *J. Pediatr*. **111**, 644–650.

Armstrong, S., Yates, S.P., and Merrill, A.R. (2002). Insight into the catalytic mechanism of Pseudomonas aeruginosa exotoxin A. Studies of toxin interaction with eukaryotic elongation factor-2. *J. Biol. Chem*. **277**, 46669–46675.

Bacha, P., Williams, D.P., Waters, C., Williams, J.M., Murphy, J.R. and Strom, T.B. (1988). Interleukin 2 receptor-targeted cytotoxicity. Interleukin 2 receptor-mediated action of a diphtheria toxin-related interleukin 2 fusion protein. *J. Exp. Med*. **167**, 612–622.

Baldwin, R.L., Kobrin, M.S., Tran, T., Pastan, I. and Korc, M. (1996). Cytotoxic effects of TGF-alpha-Pseudomonas exotoxin A fusion protein in human pancreatic carcinoma cells. *Pancreas* **13**, 16–21.

Barbieri, J.T. and Cortina, G. (1988). ADP-ribosyltransferase mutations in the catalytic S-1 subunit of pertussis toxin. *Infect. Immun*. **56**, 1934–1941.

Barbieri, J.T., Mende-Mueller, L.M., Rappuoli, R. and Collier, R.J. (1989). Photolabeling of Glu-129 of the S-1 subunit of pertussis toxin with NAD. *Infect. Immun*. **57**, 3549–3554.

Barbieri, J.T. (2000). Pseudomonas aeruginosa exoenzyme S, a bifunctional type-III secreted cytotoxin. *Int. J. Med. Microbiol*. **290**, 381–387.

Barth H., Preiss J.C., Hofmann F., and Aktories, K. (1998). Characterization of the catalytic site of the ADP-ribosyltransferase Clostridium botulinum C2 toxin by site-directed mutagenesis. *J. Biol. Chem*. **273**, 29506–11.

Barth, H., Blocker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R., and Aktories, K. (2000). Cellular uptake of Clostridium botulinum C2 toxin requires oligomerization and acidification. *J. Biol. Chem*. **275**(25):18704–11.

Bastiaens, P.I., Majoul, I.V., Verveer, P.J., Soling, H.D. and Jovin, T.M. (1996). Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *EMBO J*. **15**, 4246–4253.

Bell, C.E. and Eisenberg, D. (1997). Crystal structure of diphtheria toxin bound to nicotinamide adenine dinucleotide. *Adv. Exp. Med. Biol*. **419**, 35–43.

Bennett, M.J., Choe, S. and Eisenberg, D. (1994). Refined structure of dimeric diphtheria toxin at 2.0 angstrom resolution. *Protein Sci*. **3**, 1444–1463.

Bigio, M., Rossi, R., Nucci, D., Antoni, G., Rappuoli, R. and Ratti, G. (1987). Conformational changes in diphtheria toxoids. Analysis with monoclonal antibodies. *FEBS Lett*. **218**, 271–276.

Black, W.J., Munoz, J.J., Peacock, M.G., Schad, P.A., Cowell, J.L., Burchall, J.J., Lim, M., Kent, A., Steinman, L. and Falkow, S. (1988). ADP-ribosyltransferase activity of pertussis toxin and immunomodulation by *Bordetella pertussis*. *Science* **240**, 656–659.

Blanke, S.R., Huang, K., Wilson, B.A., Papini, E., Covacci, A. and Collier, R.J. (1994a). Active-site mutations of the diphtheria toxin catalytic domain: role of Histidine-21 in nicotinamide adenine dinucleotide binding and ADP-ribosylation of elongation factor 2. *Biochemistry* **33**, 5155–5161.

Blanke, S.R., Huang, K. and Collier, R.J. (1994b). Active-site mutations of diphtheria toxin: Role of tyrosine-65 in NAD binding and ADP-ribosylation. *Biochemistry* **33**, 15494–15500.

Blocker, D., Behlke, J., Aktories, K., and Barth, H. (2001). Cellular uptake of the Clostridium perfringens binary iota-toxin. *Infect Immun*. **69**(5):2980–7.

Blocker, D., Pohlmann, K., Haug, G., Bachmeyer, C., Benz, R., Aktories, K., and Barth, H. (2003). Clostridium botulinum C2 toxin: low pH-induced pore formation is required for translocation of the enzyme component C2I into the cytosol of host cells. *J. Biol. Chem*. **278**, 37360–7.

Boman, A.L. and Kahn, R.A. (1995). Arf proteins: the membrane traffic police? *Trends Biochem. Sci*. **20**, 147–150.

Brandhuber, B.J., Allured, V.S., Falbel, T.G. and McKay, D.B. (1988). Mapping the enzymatic active site of *Pseudomonas aeruginosa* exotoxin A. *Proteins* **3**, 146–154.

Braun, M., Stuber, K., Schlatter, Y., Wahli, T., Kuhnert, P., and Frey, J. (2002). Characterization of an ADP-ribosyltransferase toxin (AexT) from Aeromonas salmonicida subsp. salmonicida. *J. Bacteriol*. **184**, 1851–8.

Brito, G.A.C., Souza, M.H.L.P., Melo-Filho, A.A., Hewlett, E.L., Lima, A.A.M., Flores, C.A. and Ribiero, R.A. (1997). Role of pertussis toxin A subunit in neutrophil migration and vascular permeability. *Infect. Immun*. **65(3),** 1114–1118.

Brooke, J.S., Cha, J.H. and Eidels, L. (1998). Diphtheria toxin:receptor interaction: association, dissociation, and effect of pH. *Biochem. Biophys. Res. Commun*. **248**, 297–302.

Brown, B.A. and Bodley, J.W. (1979). Primary structure at the site in beef and wheat elongation factor 2 of ADP-ribosylation by diphtheria toxin. *FEBS Lett*. **103**, 253–255.

Brown, J.G., Almond, B.D., Naglich, J.G. and Eidels, L. (1993). Hypersensitivity to diphtheria toxin by mouse cells expressing both diphtheria toxin receptor and CD9 antigen. *Proc. Natl. Acad. Sci. USA* **90**, 8184–8188.

Burnette, W.N., Cieplak, W., Mar, V.L., Kaljot, K.T., Sato, H. and Keith, J.M. (1988). Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. *Science* **242**, 72–74.

Burnette, W.N., Mar, V.L., Platler, B.W., Schlotterbeck, J.D., McGinley, M.D., Stoney, K.S., Rohde, M.F. and Kaslow, H.R. (1991). Site-specific mutagenesis of the catalytic subunit of cholera toxin: substituting lysine for arginine 7 causes loss of activity. *Infect. Immun*. **59**, 4266–4270.

Burr, S.E., Stuber, K., and Frey, J. (2003). The ADP-ribosylating toxin, AexT, from Aeromonas salmonicida subsp. salmonicida is translocated via a type III secretion pathway. *J. Bacteriol*. **185**, 6583–91.

Campbell, A.M. (1962). Episomes. *Ady. Genet*. **11**, 101.

Carbonetti, N.H., Artamonova, G.V., Mays, R.M., and Worthington, Z.E. (2003). Pertussis toxin plays an early role in respiratory tract colonization by Bordetella pertussis. *Infect. Immun*. **71**, 6358–66.

Carroll, S.F., McCloskey, J.A., Crain, P.F., Oppenheimer, N.J., Marschner, T.M. and Collier, R.J. (1985). Photoaffinity labeling of diphtheria toxin fragment A with NAD: structure of the photoproduct at position 148. *Proc. Natl. Acad. Sci USA* **82**, 7237–7241.

Carroll, S.F. and Collier, R.J. (1987). Active site of *Pseudomonas aeruginosa* exotoxin A. Glutamic acid 553 is photolabeled by NAD and shows functional homology with glutamic acid 148 of diphtheria toxin. *J. Biol. Chem*. **262**, 8707–8711.

Carroll, S.F. and Collier, R.J. (1988). Amino acid sequence homology between the enzymic domains of diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A. *Mol Microbiol*. **2**, 293–296.

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989). The mammalian G-protein Rho C is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J*. **8**, 1087–1092.

Chaudhary, V.K., FitzGerald, D.J., Adhya, S. and Pastan, I. (1987). Activity of a recombinant fusion protein between transforming growth factor type alpha and Pseudomonas toxin. *Proc. Natl. Acad. Sci. USA* **84**, 4538–4542.

Chaudhary, V.K., Xu, Y.H., FitzGerald, D., Adhya, S. and Pastan, I. (1988). Role of domain II of Pseudomonas exotoxin in the secretion of proteins into the periplasm and medium by *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85**, 2939–2943.

Chaudhary, V.K., Jinno, Y., FitzGerald, D., and Pastan, I. (1990). Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. U S A*. **87**, 308–12.

Chiron, M.F., Fryling, C.M., and FitzGerald D. (1997). Furin-mediated cleavage of Pseudomonas exotoxin-derived chimeric toxins. *J. Biol. Chem*. **272**, 31707–11.

Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M., Kantardjieff, K.A., Collier, R.J. and Eisenberg. D. (1992). The crystal structure of diphtheria toxin. *Nature* **357**, 216–222.

Cieplak, W., Gaudin, H.M. and Eidels, L. (1987). Diphtheria toxin receptor. Identification of specific diphtheria toxin-binding proteins on the surface of Vero and BS-C-1 cells. *J. Biol. Chem*. **262**, 13246–13253.

Cieplak, W., Burnette, W.N., Mar, V.L., Kaljot, K.T., Morris, C.F., Chen, K.K., Sato, H. and Keith, J.M. (1988). Identification of a region in the S1 subunit of pertussis toxin that is required for enzymatic activity and that contributes to the formation of a neutralizing antigenic determinant. *Proc. Natl. Acad. Sci. USA* **85**, 4667–4671.

Coburn, J., Kane, A.V., Feig, L. and Gill, D.M. (1991). Pseudomonas aeruginosa exoenzyme S requires a eukaryotic protein for ADP-ribosyltransferase activity. *J. Biol. Chem*. **266**, 6438–6446.

Collier, R.J. (1982). Structure and activity of diphtheria toxin. In: *ADP-ribosylation reactions* (eds Hayashi, D. and Ueda, K.), p. 575. Academic Press, New York.

Comanducci, M., Ricci, S., Rappuoli, R. and Ratti, G. (1987). The nucleotide sequence of the gene coding for diphtheria toxoid CRM 176. *Nucleic Acids Res*. **15**, 5897.

Costa, J., Michel, J.L., Rappuoli, R. and Murphy, J. (1981). Restriction map of corynebacteriophages ßc and ßvir and physical localization of diphtheria tox operon. *J. Bacteriol*. **148**, 124–130.

Costantino, P., Viti, S., Podda, A., Velmonte, M.A., Nencioni, L. and Rappuoli, R. (1992). Development and phase I clinical testing of a conjugate vaccine against meningococcus A and C. *Vaccine* **10**, 691–698.

Covacci, A. and Rappuoli, R. (1993). Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. *Mol. Microbiol*. **8**, 429–434.

Coye, L.H., and Collins, C.M. (2004). Identification of SpyA, a novel ADP-ribosyltransferase of Streptococcus pyogenes. *Mol. Microbiol*. **54**, 89–98.

Dallas, W.S. and Falkow, S. (1980). Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature* 4, **288**, 499–501.

de Haan, L., Verweij, W.R., Feil, I.K., Lijnema, T.H., Hol, W.G., Agsteribbe, E. and Wilschut, J. (1996). Mutants of the *Escherichia coli* heat-labile enterotoxin with reduced ADP-ribosylation activity or no activity retain the immunogenic properties of the native holotoxin. *Infect. Immun*. **64**, 5413–5416.

Dell'Arciprete, L., Colombatti, M., Rappuoli, R. and Tridente, G. (1988). A C terminus cysteine of diphtheria toxin B chain involved in immunotoxin cell penetration and cytotoxicity. *J. Immunol*. **140**, 2466–2471.

Dickinson, B.L. and Clements, J.D. (1995). Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infect. Immun*. **63**, 1617–1623.

Ding, X., Zeng, H., Schiering, N., Ringe, D. and Murphy, J.R. (1996). Identification of the primary metal ion-activation sites of the diphtheria tox repressor by X-ray crystallography and site-directed mutational analysis. *Nat. Struct. Biol*. **3**, 382–387.

Di Tommaso, A., Saletti, G., Pizza, M., Rappuoli, R., Dougan, G., Abrignani, S., Douce, G. and De Magistris, M.T. (1996). Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. *Infect. Immun*. **64**, 974–979.

Domenighini, M., Montecucco, C. and Ripka, W.C. and Rappuoli, R. (1991). Computer modelling of the NAD binding site of ADP-ribosylating toxins: active-site structure and mechanism of NAD binding. *Mol. Microbiol*. **5**, 23–31.

Domenighini, M., Magagnoli, C., Pizza, M. and Rappuoli, R. (1994). Common features of the NAD-binding and catalytic site of ADP-ribosylating toxins. *Mol. Microbiol*. **14**, 41–50.

Domenighini, M., Pizza, M., and Rappuoli, R. (1995a). Bacterial ADP-ribosyltransferases. In: *Bacterial Toxins and Virulence Factors in Disease* (eds Moss, J., Iglewski, B., Vaughan, M., *et al*.) p. 59, Marcel Dekker, Inc., New York.

Domenighini, M., Pizza, M., Jobling, M.G., Holmes, R.K. and Rappuoli, R. (1995b). Identification of errors among database sequence entries and comparison of correct amino acid sequences for the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. *Mol. Microbiol*. **15**, 1165–1167.

Domenighini, M. and Rappuoli, R. (1996). Three conserved consensus sequences identify the NAD binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria and T-even bacteriophages. *Mol. Microbiol*. **21**, 667–674.

Donaldson, J.G. and Klausner, R.D. (1994). ARF: a key regulatory switch in membrane traffic and organelle structure. *Curr. Opin. Cell. Biol*. **6**, 527–532.

Douce, G., Turcotte, C., Cropley, I., Roberts, M., Pizza, M., Domenghini, M. and Rappuoli R. and Dougan, G. (1995). Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants. *Proc. Natl. Acad. Sci. USA* **92**, 1644–1648.

Douce, G., Fontana, M., Pizza, M., Rappuoli, R. and Dougan, G. (1997). Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. *Infect. Immun*. **65**, 2821–2828.

Douglas, C.M. and Collier, R.J. (1990). Pseudomonas aeruginosa exotoxin A: alterations of biological and biochemical properties resulting from mutation of glutamic acid 553 to aspartic acid. *Biochemistry* **29**, 5043–5049.

Egan, W., Frasch, C.E. and Anthony, B.F. (1995). Lot-release criteria, postlicensure quality control, and the *Haemophilus influenzae* type b conjugate vaccines. *JAMA* **273**, 888–889.

Essand, M. and Pastan, I. (1998). Anti-prostate immunotoxins: cytotoxicity of E4 antibody-Pseudomonas exotoxin constructs. *Int. J. Cancer* **77**, 123–127.

Evans, H.R., Sutton, J.M., Holloway, D.E., Ayriss, J., Shone, C.C., and Acharya, K.R. (2003). The crystal structure of C3stau2 from Staphylococcus aureus and its complex with NAD. *J. Biol. Chem*. **278**, 45924–30.

Falnes, P.O., Choe, S., Madshus, I.H., Wilson, B.A., and Olsnes, S. (1994). Inhibition of membrane translocation of diphtheria toxin A-fragment by internal disulfide bridges. *J. Biol. Chem*. **269**, 8402–7.

Farizo, K.M., Huang, T., and Burns, D.L. (2000). Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from Bordetella pertussis. *Infect. Immun*. **68**, 4049–54.

Farizo, K.M., Fiddner, S., Cheung, A.M., and Burns, D.L. (2002). Membrane localization of the S1 subunit of pertussis toxin in Bordetella pertussis and implications for pertussis toxin secretion. *Infect. Immun*. **70**, 1193–201.

Field, M., Rao, M.C. and Chang, E.B. (1989a). Intestinal electrolyte transport and diarrheal disease (1). *N. Engl. J. Med*. **321**, 800–806.

Field, M., Rao, M.C., and Chang, E.B. (1989b). Intestinal electrolyte transport and diarrheal disease (2). *N. Engl. J. Med*. **321**, 879–883.

Fontana, M.R., Manetti, R., Giannelli, V., Magagnoli, C., Marchini, A., Olivieri, R., Domenighini, M., Rappuoli, R. and Pizza, M. (1995). Construction of nontoxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. *Infect. Immun*. **63**, 2356–2360.

Freeman, V.J. (1951). Studies on the virulence of bacteriophage infected strains of *Corynebacterium diphtheriae*. *J. Bacteriol*. **61**, 675.

Frithz-Lindsten, E., Du, Y., Rosqvist, R. and Forsberg, A. (1997). Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity, and disruption of actin microfilaments. *Mol. Microbiol*. **25**, 1125–1139.

Fujita, H., Okamoto, H. and Tsuyama, S. (1995). ADP-ribosylation in adrenal glands: purification and characterization of mono-ADP-ribosyltransferases and ADP-ribosylhydrolase affecting cytoskeletal actin. *Int. J. Biochem. Cell. Biol*. **27**, 1065–1078.

Galloway, D.R., Hedstrom, R.C., McGowan, J.L., Kessler, S.P. and Wozniak, D.J. (1989). Biochemical analysis of CRM 66. A nonfunctional *Pseudomonas aeruginosa* exotoxin A. *J. Biol. Chem*. **264**, 14869–14873.

Ganesan, A.K., Frank, D.W., Misra, R.P., Schmidt, G. and Barbieri, J.T. (1998). *Pseudomonas aeruginosa* exoenzyme S ADP-ribosylates Ras at multiple sites. *J. Biol. Chem*. **273**, 7332–7337.

Gether, U. and Kobilka, B.K. (1998). G protein-coupled receptors. II. Mechanism of agonist activation. *J. Biol. Chem*. **273**, 17979–17982.

Ghiara, P., Rossi, M., Marchetti, M., Di Tommaso, A., Vindigni, C., Ciampolini, F., Covacci, A., Telford, J.L., De Magistris, M.T., Pizza, M., Rappuoli, R., and Del Giudice, G. (1997). Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradi-

cates an otherwise chronic infection and confers protection against reinfection. *Infect. Immun*. **65**, 4996–5002.

Giannelli, V., Fontana, M.R., Giuliani, M.M., Guangcai, D., Rappuoli, R. and Pizza, M. (1997). Protease susceptibility and toxicity of heat-labile enterotoxins with a mutation in the active site or in the protease-sensitive loop. *Infect. Immun*. **65**, 331–334.

Giannini, G., Rappuoli, R. and Ratti, G. (1984). The amino acid sequence of two nontoxic mutants of diphtheria toxin: CRM 45 and CRM 197. *Nucleic Acids Res*. **12**, 4063–4069.

Gilchrist, A., Mazzoni, M.R., Dineen, B., Dice, A., Linden, J., Proctor, W.R., Lupica, C.R., Dunwiddie, T.V., Hamm and H.E. (1998). Antagonists of the receptor-G protein interface block Gi-coupled signal transduction. *J. Biol. Chem*. **273**, 14912–14919.

Gill, D.M., Pappenheimer, A.M. Jr., Brown, R., and Kurnick, J.T. (1969). Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med*. **129**, 1–21.

Gill, D.M. and Rappaport, R.S. (1979). Origin of the enzymatically active A1 fragment of cholera toxin. *J. Infect. Dis*. **139**, 674–680.

Gilman, A.G. (1984). G proteins and dual control of adenylate cyclase. *Cell* **36**, 577–579.

Giuliani, M.M., Del Giudice, G., Giannelli, V., Dougan, G., Douce, G., Rappuoli, R. and Pizza, M. (1998). Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. *J. Exp. Med*. **187**, 1123–1132.

Glowacki, G., Braren, R., Firner, K., Nissen, M., Kuhl, M., Reche, P., Bazan, F., Cetkovic-Cvrlje, M., Leiter, E., Haag, F., and Koch-Nolte, F. (2002). The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse. *Protein Sci*. **1**,1657–1670.

Grant, C.C., Messer, R.J. and Cieplak, W. Jr. (1994). Role of trypsin-like cleavage at arginine 192 in the enzymatic and cytotonic activities of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun*. **62**, 4270–4278.

Gray, G.L., Smith, D.H., Baldridge, J.S., Harkins, R.N., Vasil, M.L., Chen, E.Y. and Heyneker, H.L. (1984). Cloning nucleotide sequence, and expression in *Escherichia coli* of the exotoxin. A structural gene of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. USA* **81**, 2645–2649.

Greenfield, L., Bjorn, M.J., Horn, G., Fong, D., Buck, G.A., Collier, R.J. and Kaplan, D.A. (1983). Nucleotide sequence of the structural gene for diphtheria toxin carried by corynephage 0. *Proc. Natl. Acad. Sci. USA* **80**, 6853–6857.

Greenfield L., Johnson, V.G. and Youle, R.J. (1987). Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity. *Science* **238**, 536–539.

Griffiths, S.L., Finkelstein, R.A. and Critchley, D.R. (1986). Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile toxin in rabbit intestinal brush borders. *Biochem. J*. **238**, 313–322.

Guidry, J.J., Cardenas, L., Cheng, E. and Clements, J.D. (1997). Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun*. **65**, 4943–4950.

Gulke, I., Pfeifer, G., Liese, J., Fritz, M., Hofmann, F., Aktories, K., and Barth, H. (2001). Characterization of the enzymatic component of the ADP-ribosyltransferase toxin CDTa from Clostridium difficile. *Infect. Immun*. **69**, 6004–6011.

Hamm, H.E. and Gilchrist, A. (1996). Heterotrimeric G proteins. *Curr. Opin. Cell. Biol*. **8**, 189–196.

Han, S., Craig, J.A., Putnam, C.D., Carozzi, N.B., and Tainer, J.A. (1999). Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nat. Struct. Biol*. **6**, 932–936.

Han, S., Arvai, A.S., Clancy, S.B., and Tainer, J.A. (2001). Crystal structure and novel recognition motif of rho ADP-ribosylating C3 exoenzyme from Clostridium botulinum: structural insights for recognition specificity and catalysis. *J. Mol. Biol*. **305**, 95–107.

Han, S., and Tainer, J.A. (2002). The ARTT motif and a unified structural understanding of substrate recognition in ADP-ribosylating bacterial toxins and eukaryotic ADP-ribosyltransferases. *Int. J. Med. Microbiol*. **291**, 523–529.

Haug, G., Aktories, K., and Barth, H. (2004). The host cell chaperone Hsp90 is necessary for cytotoxic action of the binary iota-like toxins. *Infect. Immun*. **72**, 3066–3068.

Hewlett, E.L., Sauer, K.T., Myers, G.A., Cowell, J.L. and Guerrant, R.L. (1983). Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. *Infect. Immun*. **40**, 1198–1203.

Hill, C.S., Wynne, J. and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159–1170.

Hofstra, H. and Witholt, B. (1985). Heat-labile enterotoxin in *Escherichia coli*. Kinetics of association of subunits into periplasmic holotoxin. *J. Biol. Chem*. **260**, 16037–16044.

Holmgren, J. (1973). Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoid. *Infect. Immun*. **8**, 851–859.

Holmgren, J. (1981). Actions of cholera toxin and the prevention and treatment of cholera. *Nature* **292**, 413–417.

Holmgren, J., Fredman, P., Lindblad, M., Svennerholm, A.M. and Svennerholm, L. (1982). Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. *Infect. Immun*. **38**, 424–433.

Holmgren, J., Lindblad, M., Fredman, P., Svennerholm, L. and Myrvold, H. (1985). Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. *Gastroenterology* **89**, 27–35.

Honjo, T., Nishizuka, Y. and Hayaishi, O. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem*. **243**, 3553–3555.

Hooper, K.P. and Eidels, L. (1995). Localization of a critical diphtheria toxin-binding domain to the C-terminus of the mature heparin-binding, EGF-like growth factor region of the diphtheria toxin receptor. *Biochem. Biophys. Res. Commun*. **206**, 710–717.

Horstman, A.L., and Kuehn, M.J. (2002). Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway. *J. Biol. Chem*. **277**, 32538–32545.

Hwang, J., Fitzgerald, D.J., Adhya, S. and Pastan, I. (1987). Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* **48**, 129–136.

Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1994). Heparin-binding, EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO J*. **13**, 2322–2330.

Jenkins, C.E., Swiatoniowski, A., Issekutz, A.C., and Lin, T.J. (2004). Pseudomonas aeruginosa exotoxin A induces human mast cell apoptosis by a caspase-8 and -3-dependent mechanism. *J. Biol. Chem*. **279**, 37201–37207.

Ji, T.H., Grossmann, M. and Ji, I. (1998). G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J. Biol. Chem*. **273**, 17299–17302.

Jinno, Y., Chaudhary, V.K., Kondo, T., Adhya, S., FitzGerald, D.J. and Pastan, I. (1988). Mutational analysis of domain I of Pseudomonas

exotoxin. Mutations in domain I of Pseudomonas exotoxin, which reduce cell binding and animal toxicity. *J. Biol. Chem*. **263**, 13203–13207.

Jinno, Y., Ogata, M., Chaudhary, V.K., Willingham, M.C., Adhya, S., FitzGerald, D. and Pastan, I. (1989). Domain II mutants of Pseudomonas exotoxin deficient in translocation. *J. Biol. Chem*. **264**, 15953–15959.

Johannes, L. and Goud, B. (1998). Surfing on a retrograde wave: how does Shiga toxin reach the endoplasmic reticulum? *Trends. Cell. Biol*. **8**, 158–162.

Johnson V.G., Nicholls, P.J., Habig, W.H. and Youle, R.J. (1993). The role of proline 345 in diphtheria toxin translocation. *J. Biol. Chem*. **268**, 3514–3519.

Johnson, V.G. and Nicholls, P.J. (1994a). Identification of a single amino acid substitution in the diphtheria toxin A chain of CRM 228 responsible for the loss of enzymatic activity. *J. Bacteriol*. **176**, 4766–4769.

Johnson, V.G. and Nicholls, P.J. (1994b). Histidine 21 does not play a major role in diphtheria toxin catalysis. *J. Biol. Chem*. **269**, 4349–4354.

Just, I., Mohr, C., Schallehn, G., Menard, L., Didsbury, J.R., Vandekerckhove, J., van Damme, J., and Aktories, K. (1992). Purification and characterization of an ADP-ribosyltransferase produced by Clostridium limosum. *J. Biol. Chem*. **267**, 10274–10280.

Just, I., Selzer, J., Jung, M., van Damme, J., Vandekerckhove, J., and Aktories, K. (1995). Rho-ADP-ribosylating exoenzyme from Bacillus cereus. Purification, characterization, and identification of the NAD-binding site. *Biochemistry*. **34**, 334–340.

Kaczorek, M., Delpeyroux, F., Chenciner, N., Streeck, R.E., Murphy, J.R., Boquet, P. and Tiollais, P. (1983). Nucleotide sequence and expression of the diphtheria tox 228 gene in *Escherichia coli. Science* **221**, 855–858.

Kanazawa, T., Watanabe, M., Matsushima-Hibiya, Y., Kono, T., Tanaka, N., Koyama, K., Sugimura, T., and Wakabayashi, K. (2001). Distinct roles for the N- and C-terminal regions in the cytotoxicity of pierisin-1, a putative ADP-ribosylating toxin from cabbage butterfly, against mammalian cells. *Proc. Natl. Acad. Sci. USA*. **98**, 2226–2231.

Karlsson, K.A., Teneberg, S., Angstrom, J., Kjellberg, A., Hirst, T.R., Berstrom, J. and Miller-Podraza, H. (1996). Unexpected carbohydrate cross-binding by *Escherichia coli* heat-labile enterotoxin. Recognition of human and rabbit target cell glycoconjugates in comparison with cholera toxin. *Bioorg. Med. Chem*. **11**, 1919–1928.

Kaslow, H.R., Schlotterbeck, J.D., Mar, V.L. and Burnette, W.N. (1989). Alkylation of cysteine 41, but not cysteine 200, decreases the ADP-ribosyltransferase activity of the S1 subunit of pertussis toxin. *J. Biol. Chem*. **264**, 6386–6390.

Katada, T., Tamura, M. and Ui, M. (1983). The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch. Biochem. Biophys*. **224**, 290–298.

Kjeldgaard, M., Nyborg, J. and Clark, B.F. (1996). The GTP binding motif: variations on a theme. *FASEB J*. **10**, 1347–68.

Kohno, K., Uchida, T., Ohkubo, H., Nakanishi, S., Nakanishi, T., Fukui, T., Ohtsuka, E., Ikehara, M. and Okada, Y. (1986). Amino acid sequence of mammalian elongation factor 2 deduced from the cDNA sequence: homology with GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **83**, 4978–4982.

Kosecka, U., Marshall, J. S., Crowe, S.E., Bienenstock J. and Perdue, M.H. (1994). Pertussis toxin stimulates hypersensitivity and enhances nerve-mediated antigen uptake in rat intestine. *Amer. J. Physiol. Gastrointest*. L. **30**, G745–G753.

Jobling, M.G. and Holmes, R.K. (2001). Biological and biochemical characterization of variant A subunits of cholera toxin constructed by site-directed mutagenesis. *J. Bacteriol*. **183**, 4024–32.

Laird, W. and Groman, N. (1976). Isolation and characterization of tox mutants of corynebacteriophage beta. *J. Virol*. **19**, 220–227.

Lebens, M., Johansson, S., Osek, J., Lindblad, M. and Holmgren, J. (1993). Large-scale production of *Vibrio cholerae* toxin B subunit for use in oral vaccines. *Biotechnology* **11**, 1574–1578.

Lefkowitz, R.J. (1998). G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem*. **273**, 18677–80.

Lencer, W.I., and Tsai, B. (2003). The intracellular voyage of cholera toxin: going retro. *Trends Biochem. Sci*. **28**, 639–645.

Liu, Z.X., Azhipa, O., Okamoto, S., Govindarajan, S., and Dennert, G. (2001). Extracellular nicotinamide adenine dinucleotide induces t cell apoptosis *in vivo* and *in vitro. J. Immunol*. **167**, 4942–4947.

Liu, S. and Leppla, S.H. (2003). Retroviral insertional mutagenesis identifies a small protein required for synthesis of diphthamide, the target of bacterial ADP-ribosylating toxins. *Molecular Cell*. **12**, 603–613.

Lobet, Y., Cieplak, W. Jr, Smith, S.G. and Keith, J.M. (1989). Effects of mutations on enzyme activity and immunoreactivity of the S1 subunit of pertussis toxin. *Infect. Immun*. **57**, 3660–3662.

Lobet, Y., Cluff, C.W. and Cieplak, W. Jr. (1991). Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun*. **59**, 2870–2879.

Lobet, Y., Feron, C., Dequesne, G., Simoen, E., Hauser, P. and Locht, C. (1993). Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J. Exp. Med*. **177**, 79–87.

Locht, C. and Keith, J.M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232**, 1258–1264.

Locht, C., Capiau, C. and Feron, C. (1989). Identification of amino acid residues essential for the enzymatic activities of pertussis toxin. *Proc. Natl. Acad. Sci. U S A* **86**, 3075–3079.

Lorberboum-Galski, H., FitzGerald, D., Chaudhary, V., Adhya, S. and Pastan, I. (1988). Cytotoxic activity of an interleukin 2-Pseudomonas exotoxin chimeric protein produced in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **85**, 1922–1926.

Lycke, N., Tsuji, T. and Holmgren, J. (1992). The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. *Eur. J. Immunol*. **22**, 2277–2281.

Majoul, I.V., Bastiaens, P.I. and Soling, H.D. (1996). Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J. Cell. Biol*. **133**, 777–789.

Marchetti, M., Rossi, M., Giannelli, V., Giuliani, M.M., Pizza, M., Censini, S., Covacci, A., Massari, P., Pagliaccia, C., Manetti, R., Telford, J.L., Douce, G., Dougan, G., Rappuoli, R. and Ghiara, P. (1998). Protection against *Helicobacter pylori* infection in mice by intragastric vaccination with *H. pylori* antigens is achieved using a non-toxic mutant of *E. coli* heat-labile enterotoxin (LT) as adjuvant. *Vaccine* **16**, 33–37.

Marsischky, C.T., Wilson, B.A. and Collier, R.J. (1995). Role of glutamic acid 988 of human poly-ADP-ribose polymerase in polymer formation. Evidence for active site similarities to the ADP-ribosylating toxins. *J. Biol. Chem*. **270**, 3247–3254.

Marvaud, J.C., Smith, T., Hale, M.L., Popoff, M.R., Smith, L.A., and Stiles, B.G. (2001). Clostridium perfringens iota-toxin: mapping of receptor binding and Ia docking domains on Ib. *Infect. Immun*. **69**, 2435–2441.

Masignani, V., Balducci, E., Di Marcello, F., Savino, S., Serruto, D., Veggi, D., Bambini, S., Scarselli, M., Arico, B., Comanducci, M., Adu-Bobie, J., Giuliani, M.M., Rappuoli, R. and Pizza, M. (2003). NarE: a novel ADP-ribosyltransferase from Neisseria meningitidis. *Mol Microbiol*. **50**, 1055–1067.

McMahon, K.K., Piron, K.J., Ha, V.T. and Fullerton, A.T. (1993). Developmental and biochemical characteristics of the cardiac membrane-bound, arginine-specific mono-ADP-ribosyltransferase. *Biochem. J.* **293**, 789–793.

Merritt, E.A., Sarfaty, S., Pizza, M., Domenighini, M., Rappuoli, R. and Hol, W.G. (1995). Mutation of a buried residue causes loss of activity but no conformational change in the heat-labile enterotoxin of *Escherichia coli*. *Nat. Struct. Biol*. **2**, 269–272.

Mitamura, T., Higashiyama, S., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding, EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem*. **270**, 1015–1019.

Mori, T., Shoemaker, R.H., McMahon, J.B., Gulakowski, R.J., Gustafson, K.R. and Boyd, M.R. (1997). Construction and enhanced cytotoxicity of a [cyanovirin-N]-[Pseudomonas exotoxin] conjugate against human immunodeficiency virus-infected cells. *Biochem. Biophys. Res. Commun*. **239**, 884–888.

Moss, J. and Vaughan, M. (1988). Cholera toxin and *E. coli* enterotoxins and their mechanisms of action. In: *Handbook of Natural Toxins*, vol 4, *Bacterial Toxins* (eds. M. Hardegree and A.T. Ru), pp. 39–87, Marcel Dekker, New York.

Moss, J., Stanley, S.J., Vaughan, M. and Tsuji, T. (1993). Interaction of ADP-ribosylation factor with *Escherichia coli* enterotoxin that contains an inactivating lysine 112 substitution. *J. Biol. Chem*. **268**, 6383–6387.

Moss, J. and Vaughan, M. (1995). Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J. Biol. Chem*. **270**, 12327–12330.

Mueller-Dieckmann, C., Ritter, H., Haag, F., Koch-Nolte, F., and Schulz, G.E. (2002). Structure of the ecto-ADP-ribosyl transferase ART2.2 from rat. *J. Mol. Biol*. **322**, 687–696.

Murphy, J.R., Bishai, W., Borowski, M., Miyanohara, A., Boyd, J. and Nagle, S. (1986). Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria, toxin-related, alpha-melanocyte-stimulating hormone fusion protein. *Proc. Natl. Acad. Sci. USA* **83**, 8258–8262.

Nagahama, M., Sakaguchi, Y., Kobayashi, K., Ochi, S. and Sakurai, J. (2000). Characterization of the enzymatic component of Clostridium perfringens iota-toxin. *J. Bacteriol*. **182**(8):2096–103.

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding, EGF-like growth factor precursor. *Cell* **69**, 1051–1061.

Nashar, T.O., Webb, H.M., Eaglestone, S., Williams, N.A. and Hirst, T.R. (1996). Potent immunogenicity of the B subunits of *Escherichia coli* heat-labile enterotoxin: receptor binding is essential and induces differential modulation of lymphocyte subsets. *Proc. Natl. Acad. Sci. USA* **93**, 226–230.

Neer, E.J. and Clapham, D.E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

Nencioni, L., Pizza, M., Bugnoli, M., De Magistris, T., Di Tommaso, A., Giovannoni, F., Manetti, R., Marsili, I., Matteucci, G., Nucci, D., Olivieri, R., Pileri, P., Presentini, R., Villa, L., Kreeftenberg, H., Silvestri, S., Tagliabue, A. and Rappuoli, R. (1990). Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. *Infect. Immun*. **58**, 1308–1315.

Nencioni, L., Volpini, G., Peppoloni, S., de Magistris, M.T., Marsili, I. and Rappuoli, R. (1991). Properties of the pertussis toxin mutant PT-9K/129G after formaldehyde treatment. *Infect. Immun*. **59**, 625–630.

Neville, D.M. Jr. and Hudson, T.H. (1986). Transmembrane transport of diphtheria toxin, related toxins, and colicins. *Annu. Rev. Biochem*. **55**, 195–224.

Nicosia, A., Perugini, M., Franzini, C., Casagli, M.C., Borri, M.G., Antoni, G., Almoni, M., Neri, P., Ratti, G. and Rappuoli, R. (1986). Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proc. Natl. Acad. Sci. USA* **83**, 4631–4635.

Ogata, M., Chaudhary, V.K., FitzGerald, D.J. and Pastan, I. (1989). Cytotoxic activity of a recombinant fusion protein between interleukin 4 and Pseudomonas exotoxin. *Proc. Natl. Acad. Sci. USA* **86**, 4215–4219.

Okamoto, K., Okamoto, K., Miyama, A., Tsuji, T., Honda, T. and Miwatani, T. (1988). Effect of substitution of glycine for arginine at position 146 of the A1 subunit on biological activity of *Escherichia coli* heat-labile enterotoxin. *J. Bacteriol*. **170**, 2208–2211.

Okazaki, I.J. and Moss, J. (1994). Common structure of the catalytic sites of mammalian and bacterial toxin ADP-ribosyltransferases. *Mol. Cell. Biochem*. **138**, 177–181.

Okazaki, I.J. and Moss, J. (1998). Glycosylphosphatidylinositol-anchored and secretory isoforms of mono-ADP-ribosyltransferases. *J Biol Chem*. **273**, 23617–23620.

O'Neal, C.J., Amaya, E.I., Jobling, M.G., Holmes, R.K., and Hol, W.G. (2004). Crystal structures of an intrinsically active cholera toxin mutant yield insight into the toxin activation mechanism. *Biochemistry*. **43**, 3772–82.

Otto, H., Tezcan-Merdol, D., Girisch, R., Haag, F., Rhen, M., and Koch-Nolte, F. (2000). The spvB gene-product of the Salmonella enterica virulence plasmid is a mono (ADP-ribosyl) transferase. *Mol. Microbiol*. **37**, 1106–1115.

Pallen, M.J., Lam, A.C., Loman, N.J., and McBride, A. (2001). An abundance of bacterial ADP-ribosyltransferases—implications for the origin of exotoxins and their human homologues. *Trends Microbiol*. **9**, 302–307.

Paone, G., Wada, A., Stevens, L.A., Matin, A., Hirayama, T., Levine, R.L. and Moss, J. (2002) ADP ribosylation of human neutrophil peptide-1 regulates its biological properties. *Proc. Natl. Acad. Sci. USA*. **99**, 8231–8235.

Papini, E., Colonna, R., Cusinato, F., Montecucco, C., Tomasi, M. and Rappuoli, R. (1987a). Lipid interaction of diphtheria toxin and mutants with altered fragment B. 1. Liposome aggregation and fusion. *Eur. J. Biochem*. **169**, 629–635.

Papini, E., Schiavo, G., Tomasi, M., Colombatti, M., Rappuoli, R. and Montecucco, C. (1987b). Lipid interaction of diphtheria toxin and mutants with altered fragment B. 2. Hydrophobic photolabelling and cell intoxications. *Eur. J. Biochem*. **169**, 637–644.

Papini, E., Schiavo, G., Sandona, D., Rappuoli, R. and Montecucco, C. (1989). Histidine 21 is at the NAD+ binding site of diphtheria toxin. *J. Biol. Chem*. **264**, 12385–12388.

Papini, E., Santucci, A., Schiavo, G., Domenighini, M., Neri, P., Rappuoli, R. and Montecucco, C. (1991). Tyr-65 is photolabeled by 8-azido adenine and 8-azido-adenosine at the NAD binding site of diphtheria toxin. *J. Biol. Chem*. **266**, 2494–2498.

Pappenheimer, A.M. (1938). Diphtheria toxin. II. The action of ketene and formaldehyde. *J. Biol. Chem*. **125**, 201–208.

Pappenheimer, A.M. Jr., Uchida, T. and Harper, A.A. (1972). An immunological study of the diphtheria toxin molecule. *Immunochemistry* **9**, 891–906.

Pappenheimer, A.M. Jr. (1977). Diphtheria toxin. *Annu. Rev. Biochem*. **46**, 69–94.

Pappenheimer, A.M. Jr (1984). Diphtheria. In: *Bacterial Vaccines* (ed. R. Germanier), pp. 1–16. Academic Press, New York.

Partidos, C.D., Pizza, M., Rappuoli, R. and Steward, M.W. (1996). The adjuvant effect of a non-toxic mutant of heat-labile enterotoxin of *Escherichia coli* for the induction of measles virus-specific CTL responses after intranasal co-immunization with a synthetic peptide. *Immunology* **89**, 483–487.

Pastan, I. and FitzGerald, D. (1989). Pseudomonas exotoxin: chimeric toxins. *J. Biol. Chem*. **264**, 15157–15160.

Perelle, S., Gibert, M., Boquet, P. and Popoff, M.R. (1993). Characterization of Clostridium perfringens Iota-toxin genes and expression in *Escherichia coli. Infect. Immun*. **63**, 5147–5156.

Pizza, M., Bartoloni, A., Prugnola, A., Silvestri, S. and Rappuoli, R. (1988). Subunit S1 of pertussis toxin: mapping of the regions essential for ADP-ribosyltransferase activity. *Proc. Natl. Acad. Sci. USA* **85**, 7521–7525.

Pizza, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., De Magistris, M.T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M. *et al.,* (1989). Mutants of pertussis toxin suitable for vaccine development. *Science* **246**, 497–500.

Pizza, M., Bugnoli, M., Manetti, R., Covacci, A. and Rappuoli, R. (1990). The subunit S1 is important for pertussis toxin secretion. *J. Biol. Chem*. **265**, 17759–17763.

Pizza, M., Domenighini, M., Hol, W., Giannelli, V., Fontana, M.R., Giuliani, M.M., Magagnoli, C., Peppoloni, S., Manetti, R. and Rappuoli, R. (1994a). Probing the structure-activity relationship of *Escherichia coli* LT-A by site-directed mutagenesis. *Mol. Microbiol*. **14**, 51–60.

Pizza, M., Giuliani, M.M., Fontana, M.R., Monaci, E., Douce, G., Dougan, G., Mills, K.H., Rappuoli, R., and Del Giudice, G. (2001). Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine*. **19**, 2534–41.

Pizza, M., Fontana, M.R., Giuliani, M.M., Domenighini, M., Magagnoli, C., Giannelli, V., Nucci, D., Hol, W., Manetti, R. and Rappuoli, R. (1994b). A genetically detoxified derivative of heat-labile *Escherichia coli* enterotoxin induces neutralizing antibodies against the A subunit. *J. Exp. Med*. **180**, 2147–2153.

Pohl, E., Holmes, R.K. and Hol, W.G. (1998). Motion of the DNA-binding domain with respect to the core of the diphtheria toxin repressor (DtxR) revealed in the crystal structures of apo- and holo-DtxR. *J. Biol. Chem*. **273**, 22420–22427.

Popoff, M.R., Rubin, E.J., Gill, D.M., and Boquet, P. (1988). Actin-specific ADP-ribosyltransferase produced by a Clostridium difficile strain. *Infect. Immun*. **56**, 2299–2306.

Porro, M., Saletti, M., Nencioni, L., Tagliaferri, L. and Marsili, I. (1980). Immunogenic correlation between cross-reacting material (CRM 197) produced by a mutant of *Corynebacterium diphtheriae* and diphtheria toxoid. *J. Infect. Dis*. **142**, 716–724.

Qiu, X., Verlinde, C.L., Zhang, S., Schmitt, M.P., Holmes, R.K. and Hol, W.G. (1995). Three-dimensional structure of the diphtheria toxin repressor in complex with divalent cation co-repressors. *Structure* **3**, 87–100.

Radke, J., Pederson, K.J., and Barbieri, J.T. (1999). Pseudomonas aeruginosa exoenzyme S is a biglutamic acid ADP-ribosyltransferase. *Infect. Immun*. **67**, 1508–1510.

Rappuoli, R. (1983). Isolation and characterization of *Corynebacterium diphtheriae* nontandem double lysogens hyperproducing CRM 197. *Appl. Enzy. Microbiol*. **45**, 560–564.

Rappuoli, R., Michel, J.L. and Murphy, J.R. (1983a). Restriction endonuclease map of corynebacteriophage ωctox+ isolated from the Park Williams no. 8 strain of *Corynebacterium diphtheriae. J. Virol*. **45**, 524–530.

Rappuoli, R., Michel, J.L. and Murphy, J.R. (1983b). Integration of corynebacterophages ßtox+, ωtox+, and γtox- into two attachment sites on the *Corynebacterium diphtheriae* chromosome. *J. Bacteriol*. **153**, 1202–1210.

Rappuoli, R. and Ratti, G. (1984). Physical map of the chromosomal region of *Corynebacterium diphtheriae* containing corynephage attachment sites attB l and attB2. *J. Bacteriol*. **158**, 325–30.

Rappuoli, R. (1997). Rational design of vaccines. *Nat. Med*. **3**, 374–376.

Ratti, G., Rappuoli, R. and Giannini, G. (1983). Complete nucleotide sequence of the gene coding for diphtheria toxin in the corynephage omegatox+ genome. *Nucleic Acids Res*. **11**, 6589–6595.

Ratts, R., Zeng, H., Berg, E.A., Blue, C., McComb, M.E., Costello, C.E., vanderSpek, J.C., and Murphy, J.R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J. Cell Biol*. **160**(7):1139–50.

Ricci, S., Rappuoli, R. and Scarlato, V. (1996). The pertussis toxin liberation genes of Bordetella pertussis are transcriptionally linked to the pertussis toxin operon. *Infect. Immun*. **64**, 1458–1460.

Roberts, M., Bacon, A., Rappuoli, R., Pizza, M., Cropley, I., Douce, G., Dougan, G., Marinaro, M., McGhee, J. and Chatfield, S. (1995). A mutant pertussis toxin molecule that lacks ADP-ribosyltransferase activity, PT-9K/129G, is an effective mucosal adjuvant for intranasally delivered proteins. *Infect. Immun*. **63**, 2100–2108.

Rothbrock, G., Smithee, L., Rados, M. and Baughman, W. (1995). Progress toward elimination of *Haemophilus influenzae* type b disease among infants and children—United States, 1993–1994 (Reprinted from MMWR, vol 44, pg 545–550, 1995). *JAMA* **274**, 1334.

Ruf, A., Mennissier de Murcia, J., de Murcia, G. and Schulz, G.E. (1996). Structure of the catalytic fragment of poly(AD-ribose) polymerase from chicken. *Proc. Natl. Acad. Sci. USA* **93**, 7481–7485.

Sandvig, K. and Olsnes, S. (1981). Rapid entry of nicked diphtheria toxin into cells at low pH. Characterization of the entry process and effects of low pH on the toxin molecule. *J. Biol. Chem*. **256**, 9068–9076.

Saxty, B.A. and van Heyningen, S. (1995). The purification of a cysteine-dependent NAD+ glycohydrolase activity from bovine erythrocytes and evidence that it exhibits a novel ADP-ribosyltransferase activity. *Biochem. J.* **310**, 931–937.

Schirmer, J., Just, I., and Aktories, K. (2002) The ADP-ribosylating mosquitocidal toxin from Bacillus sphaericus: proteolytic activation, enzyme activity, and cytotoxic effects. *J. Biol. Chem*. **277**, 11941–11948. Epub 2002 Jan 25.

Schuman, E.M., Meffert, M.K., Schulman, H. and Madison, D.V. (1994). An ADP-ribosyltransferase as a potential target for nitric oxide action in hippocampal long-term potentiation. *Proc. Natl. Acad. Sci. USA* **91**, 11958–11962.

Sekine, A., Fujiwara, M. Narumiya, S. (1989). Asparagine residue in the *rho* gene product is the modification site for botulinum ADP-ribosyl transferase. *J. Biol. Chem*. **264**, 8602–8605.

Sekura, R.D., Moss, J. and Vaughan, M. (1985). In *Pertussis Toxin*. Academic Press, New York.

Siegall, C.B., Chaudhary, V.K., FitzGerald, D.J. and Pastan, I. (1988). Cytotoxic activity of an interleukin 6-Pseudomonas exotoxin fusion protein on human myeloma cells. *Proc. Natl. Acad. Sci. USA* **85**, 9738–9742.

Siegall, C.B., Chaudhary, V.K., FitzGerald, D.J. and Pastan, I. (1989). Functional analysis of domains II, Ib, and III of Pseudomonas exotoxin. *Biol. Chem*. **264**, 14256–14261.

Silverman, J.A., Mindell, J.A., Finkelstein, A., Shen, W.H. and Collier, R.J. (1994). Mutational analysis of the helical hairpin region of diphtheria toxin transmembrane domain. *J. Biol. Chem*. **269**, 22524–22532.

Sindt, K.A., Hewlett, E.L., Redpath, G.T., Rappuoli, R., Gray, L.S. and Vandenberg, S. R.. (1994). Pertussis toxin activates platelets through an interaction with platelet glycoprotein Ib. *Infect. Immun*. **62**, 3108–3114.

Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., van Zanten, B.A., Witholt. B. and Hol, W.G. (1991). Crystal structure of a cholera toxin-related, heat-labile enterotoxin from *E. coli. Nature* **351**, 371–377.

Sixma, T.K., Kalk, K.H., van Zanten, B.A., Dauter, Z., Kingma, J., Witholt, B. and Hol, W.G. (1993). Refined structure of *Escherichia*

*coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol*. **230**, 890–918.

Spicer, E.K., Kavanaugh, W.M., Dallas, W.S., Falkow, S., Konigsberg, W.H. and Schafer DE. (1981). Sequence homologies between A subunits of *Escherichia coli* and *Vibrio cholerae* enterotoxins. *Proc. Natl. Acad. Sci. USA* **78**, 50–54.

Stein, P.E., Boodhoo, A., Armstrong, G.D., Heerze, L.D., Cockle, S.A., Klein, M.H. and Read, R.J. (1994a). Structure of a pertussis toxin-sugar complex as a model for receptor binding. *Nat. Struct. Biol*. **1**, 591–596.

Stein, P.E., Boodhoo, A., Armstrong, G.D., Cockle, S.A., Klein, M.H. and Read, R.J. (199b). The crystal structure of pertussis toxin. *Structure* **2**, 45–57.

Stevens, L.A., Moss, J., Vaughan, M., Pizza, M. and Rappuoli, R. (1999). Effects of site-directed mutagenesis of *Escherichia coli*, heat-labile enterotoxin on ADP-ribosyltransferase activity and interaction with ADP-ribosylation factors. *Infect. Immun*. **67**, 259–265.

Stiles, B.G., and Wilkins, T.D. (1986). Purification and characterization of Clostridium perfringens iota toxin: dependence on two nonlinked proteins for biological activity. *Infect. Immun*. **54**, 683–688.

Stryer, L. and Bourne, H.R. (1986). G proteins: a family of signal transducers. *Annu. Rev. Cell. Biol*. **2**, 391–419.

Sugai, M., Hashimoto, K., Kikuchi, A., Inoue, S., Okumura, H., Matsumoto, K., Goto, Y., Ohgai, H., Moriishi, K, and Syuto, B. (1992). Epidermal cell differentiation inhibitor ADP-ribosylates small GTP-binding proteins and induces hyperplasia of epidermis. *J. Biol. Chem*. **267**, 2600–2604.

Sun, J. and Barbieri, J.T. (2003) *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 regulator of kinase (Crk) proteins. *J. Biol. Chem*. **278**, 32794–32800

Sun, J., Maresso, A.W., Kim, J.J., and Barbieri, J.T. (2004). How bacterial ADP-ribosylating toxins recognize substrates. *Nat Struct Mol Biol*. **11**, 868–876.

Sunahara, R.K., Tesmer, J.J., Gilman, A.G. and Sprang, S.R. (1997). Crystal structure of the adenylyl cyclase activator Gsalpha. *Science* **278**, 1943–1947.

Sundin, C., Henriksson, M.L., Hallberg, B., Forsberg, A. and Frithz-Lindsten, E. (2001). Exoenzyme T of Pseudomonas aeruginosa elicits cytotoxicity without interfering with Ras signal transduction. *Cell Microbiol*. **3**, 237–246.

Sundin, C., Hallberg, B. and Forsberg, A. (2004). ADP-ribosylation by exoenzyme T of Pseudomonas aeruginosa induces an irreversible effect on the host cell cytoskeleton *in vivo*. *FEMS Microbiol Lett*. **234**, 87–91.

Takada, T., iida, K. and Moss, J. (1995). Conservation of a common motif in enzymes catalyzing ADP-ribose transfer. Identification of domains in mammalian transferases. *J. Biol. Chem*. **270**, 541–544.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry* **21**, 5516–5522.

Tamura, M., Nogimori, K., Yajima, M., Ase, K. and Ui, M. (1983). A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *Biol. Chem*. **258**, 6756–6761.

Tamura, S., Funato, H., Nagamine, T., Aizawa, C. and Kurata, T. (1989). Effectiveness of cholera toxin B subunit as an adjuvant for nasal influenza vaccination despite pre-existing immunity to CTB. *Vaccine* **7**, 503–505.

Tanuma, S., Kawashima, K. and Endo, H. (1988). Eukaryotic mono (ADP-ribosyl) transferase that ADP-ribosylates GTP-binding regulatory Gi protein. *J. Biol. Chem*. **263**, 5485–5489.

Teneberg, S., Hirst, T.R., Angstrom, J. and Karlsson, K.A. (1994). Comparison of the glycolipid-binding specificities of cholera toxin and porcine *Escherichia coli* heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. *Glycoconj. J*. **11**, 533–540.

Trucco, C., Flatter, E., Fribourg, S., de Murcia, G. and Menissier-de Murcia, J. (1996). Mutations in the amino-terminal domain of the human poly (ADP-ribose) polymerase that affect its catalytic activity but not its DNA binding capacity. *FEBS Lett*. **399**, 313–316.

Truitt, R.L., Hanke, C., Radke, J., Mueller, R. and Barbieri, J.T. (1998). Glycosphingolipids as novel targets for T-cell suppression by the B subunit of recombinant heat-labile enterotoxin. *Infect. Immun*. **66**, 1299–1308.

Tsai, S.C., Adamik, R., Moss, J. and Aktories, K. (1988). Separation of the 24 kDa substrate for botulinum C3 ADP-ribosyltransferase and the cholera toxin ADP-ribosylation factor. *Biochem. Biophys. Res. Commun*. **152**, 957–961.

Tsuge, H., Nagahama, M., Nishimura, H., Hisatsune, J., Sakaguchi, Y., Itogawa, Y., Katunuma, N. and Sakurai, J. (2003). Crystal structure and site-directed mutagenesis of enzymatic components from Clostridium perfringens iota-toxin. *J. Mol. Biol*. **325**, 471–483.

Tsuji, T., Inoue, T., Miyama, A., Okamoto, K., Honda, T. and Miwatani, T. (1990). A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. *J. Biol. Chem*. **265**, 22520–22525.

Tsuji, T., Inoue, T., Miyama, A. and Noda, M. (1991). Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* is important for ADP-ribosyltransferase activity. *FEBS Lett*. **291**, 319–321.

Tweten, R.K., Barbieri, J.T. and Collier, R.J. (1985). Diphtheria toxin. Effect of substituting aspartic acid for glutamic acid 148 on ADP-ribosyltransferase activity. *J. Biol. Chem*. **260**, 10392–10394.

Uchida, T., Gill, D.M. and Pappenheimer, A.M. Jr. (1971). Mutation in the structural gene for diphtheria toxin carried by temperate phage a. *Nature New. Biol*. **233**, 8–11.

Uchida, T., Pappenheimer, A.M. Jr. and Greany, R. (1973a). Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem*. **248**, 3838–3844.

Uchida, T., Pappenheimer, A.M. Jr. and Harper, A.A. (1973b). II. Kinetic studies on intoxication of HeLa cells by diphtheria toxin and related proteins. *J. Biol. Chem*. **248**, 3845–3850.

Uchida, T., Pappenheimer, A.M. Jr. and Harper, A.A. (1973c). Diphtheria toxin and related proteins. III. Reconstruction of hybrid "diphtheria toxin" from nontoxic mutant proteins. *J. Biol. Chem*. **248**, 3851–3854.

Ueda, K. and Hayaishi, O. (1985). ADP-ribosylation. *Annu. Rev. Biochem*. **54**, 73–100.

Vandekerckhove, J., Schering, B., Bärmann, M. and Aktories, K. (1988). Botulinum C2 toxin ADP-ribosylates cytoplasmic b/v-actin in arginine 177. *J. Biol. Chem*. 263, 13739–13742.

van den Akker, F., Merritt, E.A., Pizza, M., Domenighini, M., Rappuoli, R. and Hol, W.G. (1995). The Arg7Lys mutant of heat-labile enterotoxin exhibits great flexibility of active site loop 47–56 of the A subunit. *Biochemistry* **34**, 10996–11004.

van den Akker, F., Pizza, M., Rappuoli, R. and Hol, W.G. (1997). Crystal structure of a non-toxic mutant of heat-labile enterotoxin, which is a potent mucosal adjuvant. *Protein Sci*. **12**, 2650–2654.

Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980a). ADP-ribosylation of elongation factor 2 by diphtheria toxin. NMR spectra and proposed structures of ribosyl-diphthamide and its hydrolysis products. *J. Biol. Chem*. **255**, 10710–10716.

Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980b). ADP-ribosylation of elongation factor 2 by diphtheria toxin. Isolation and properties of the novel ribosyl-amino acid and its hydrolysis products. *J. Biol. Chem*. **255**, 10717–10720.

Vaughan, M. (1998). G protein-coupled receptors minireview series. *J. Biol. Chem*. **273**, 17297.

van der Pouw-Kraan, T., Rensink, I., Rappuoli, R. and Aarden, L. (1995). Co-stimulation of T cells via CD28 inhibits human IgE production. Reversal by pertussis toxin. *Clin. Exp. Immunol*. **99**, 473–478.

Wang, J., Nemoto, E., Kots, A.Y., Kaslow, H.R. and Dennert, G. (1994). Regulation of cytotoxic T cells by ecto-nicotinamide adenine dinucleotide (NAD) correlates with cell surface GPI-anchored/arginine ADP-ribosyltransferase. *J. Immunol*. **153**, 4048–4058.

Watanabe, M., Takamura-Enya, T., Kanazawa, T., Totsuka, Y., Matsushima-Hibiya, Y., Koyama, K., Sugimura, T. and Wakabayashi, K. (2002) Mono(ADP-ribosyl)ation of DNA by apoptosis-inducing protein, pierisin. *Nucleic. Acids. Res. Suppl*. **2**, 243–244.

Weiss, A.A., Johnson, F.D. and Burns, D.L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA* **90**, 2970–2974.

West, R.E., Jr., Moss, J., Vaughan, M., Liu, T. and Liu, T.Y. (1985). Pertussis toxin-catalyzed ADP-ribosylation of transducin. Cysteine 347 is the ADP-ribose acceptor site. *J. Biol. Chem*. **260**, 14428–14430.

White, A., Ding, X., vanderSpek, J.C., Murphy, J.R. and Ringe, D. (1998). Structure of the metal-ion-activated diphtheria toxin repressor/tox operator complex. *Nature* **394**, 502–506.

Wolff, C., Wattiez, R., Ruysschaert, J.M., and Cabiaux, V. (2004). Characterization of diphtheria toxin's catalytic domain interaction with lipid membranes. *Biochim. Biophys. Acta*. **1661**, 166–177.

Wiedlocha, A., Madshus, I.H., Mach, H., Middaugh, C.R. and Olsnes, S. (1992). Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. *EMBO J*. **11**, 4835–4842.

Williams, D.P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T.B. and Murphy, J.R (1987). Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. *Protein Eng*. **1**, 493–498.

Wilson, A.D., Robinson, A., Irons, L. and Stokes, C.R. (1993). Adjuvant action of cholera toxin and pertussis toxin in the induction of IgA antibody response to orally administered antigen. *Vaccine* **11**, 113–118.

Wilson, B.A., Blanke, S.R., Reich, K.A. and Collier, R.J. (1994). Active-site mutations of diphtheria toxin—Tryptophan 50 ia a major determinant of NAD affinity. *J. Biol. Chem*. **269**, 23296–23301.

Witvliet, M.H., Burns, D.L., Brennan, M.J., Poolman, J.T. and Manclark, C.R. (1989). Binding of pertussis toxin to eucaryotic cells and glycoproteins. *Infect. Immun*. **57**, 3324–3330.

Wurtele, M., Renault, L., Barbieri, J.T., Wittinghofer, A. and Wolf, E. (2001). Structure of the ExoS GTPase activating domain. *FEBS Lett*. **491**, 26–29.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978) One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15**, 245–250.

Yamamoto, S., Kiyono, H., Yamamoto, M., Imaoka, K., Fujihashi, K., Van Ginkel, F.W., Noda, M., Takeda, Y. and McGhee, J.R. (1997). A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA* **94**, 5267–5272.

Zimmermann, S., Wels, W., Froesch, B.A., Gerstmayer, B., Stahel, R.A. and Zangemeister-Wittke, U. (1997). A novel immunotoxin recognizing the epithelial glycoprotein-2 has potent antitumoral activity on chemotherapy-resistant lung cancer. *Cancer Immunol. Immunother*. **44**, 1–9.

Zolkiewska, A. and Moss, J. (1993). Integrin alpha 7 as substrate for a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase on the surface of skeletal muscle cells. *J. Biol. Chem*. **268**, 25273–25276.

Zumbihl, R., Dornand, J., Fischer, T., Cabane, S., Rappuoli, R., Bouaboula, M., Casellas, P. and Rouot, B. (1995). IL-1 stimulates a diverging signaling pathway in EL4 6.1 thymoma cells. *J. Immunol*. **155**, 181–189.

CHAPTER

# 13

# Diphtheria toxin

*Diana Marra Oram and Randall K. Holmes*

## INTRODUCTION

Diphtheria is a serious respiratory disease characterized by the formation of a pseudomembrane consisting of necrotic tissue and bacteria. *Corynebacterium diphtheriae*, visualized in 1883 by Klebs in stained samples from pseudomembranes, was isolated in 1884 by Loeffler and shown to be the cause of diphtheria. In causing respiratory diphtheria, *C. diphtheriae* colonizes the mucosus membranes of the upper respiratory tract and secretes diphtheria toxin. *C. diphtheriae* is rarely found outside of the local area of infection, but diphtheria toxin is absorbed into the circulatory system and disseminated. Many cell types are susceptible to killing by diphtheria toxin, but the primary systemic effects occur in the heart and peripheral nerves. The major clinical manifestations of diphtheria are attributable to the action of diphtheria toxin.

Diphtheria is easily transmitted from person to person and was a leading cause of infectious disease morbidity and mortality until the early 1900s. The discovery that treatment of diphtheria toxin with formaldehyde converted it to a nontoxic form (toxoid) that can still induce the formation of toxin-neutralizing antibodies provided the basis for widespread immunization against diphtheria (Ramon Jr., 1923), and immunization with toxoid has made diphtheria a rare disease in countries with high levels of medical care (Holmes, 2005). Outbreaks of diphtheria can still occur when immunity levels within a population decline. In 1990 a large outbreak of diphtheria began in Russia and quickly spread to the newly independent states of the former Soviet Union, resulting in over 150,000 cases by the mid 1990s. The outbreak was controlled by a massive immunization campaign, but not before it had resulted in more than 4,000 deaths. Small but significant outbreaks of diphtheria have also occurred in the USA, Australia, and western Europe in the last 40 years (Chen *et al*., 1985; Dittmann *et al*., 2000; Gilbert, 1997; Harnisch *et al*., 1989; Rappuoli *et al*., 1988). These outbreaks as well as recent reports that the majority of the adult population in Europe and the United States lacks adequate immunity to diphtheria has resulted in renewed interest in *C. diphtheriae*.

Diphtheria toxin was discovered in the late 1800s (Roux and Yersin, 1888) and is one of the most extensively studied bacterial toxins. In 1957 two groups reported that low concentrations of diphtheria toxin were lethal for many cell lines (Lennox and Kaplan, 1957; Placido Sousa and Evans, 1957). In 1959, Strauss and Hendee reported that the first effect of diphtheria toxin on HeLa cells was a cessation of protein synthesis at approximately one and a half hours after exposure, and other toxic effects on respiration, nucleic acid synthesis, and cellular morphology were not observed until much longer after exposure to diphtheria toxin (Strauss and Hendee, 1959). The minimal lethal dose of diphtheria toxin for humans and highly susceptible animals is below 0.1 μg per kg of body weight (Pappenheimer, 1984), and delivery of a single molecule of diphtheria toxin fragment A (the portion of the molecule containing the catalytic activity) to the cytosol is sufficient to kill a eukaryotic cell (Yamaizumi *et al*., 1978). Later, it was definitively established, by several groups, that diphtheria toxin is a specific inhibitor of eukaryotic protein synthesis.

Diphtheria toxin was the first A-B type toxin to be characterized, and it serves as the prototype for this large group of protein toxins, which includes among others, cholera toxin, *Escherichia coli* heat-labile

enterotoxin, *Pseudomonas aeruginosa* exotoxin A, tetanus and botulinum neurotoxins, and Shiga toxin. A-B type toxins have two functionally distinct components: A, the moiety representing the catalytic (toxic) function and B, the moiety representing the receptor binding function. The A and B components of A-B type toxins can be represented either by separate polypeptide subunits or by different domains of the same polypeptide, as is the case for diphtheria toxin. In *Corynebacterium diphtheriae*, diphtheria toxin is secreted from the bacterial cells as a 535 amino acid proenzyme that must be cleaved into the A fragment and the B fragment in order for its toxicity to be expressed.

## GENETICS AND REGULATION OF DIPHTHERIA TOXIN PRODUCTION

Diphtheria toxin production by *C. diphtheriae* is controlled by bacteriophage conversion (Barksdale and Pappenheimer, 1954; Freeman, 1951; Groman, 1953). Analysis of recombination between the toxin-positive corynephage β and the toxin-negative corynephage γ demonstrated that the $tox^+$ and $tox^-$ markers segregated as alleles of a unique genetic locus (Holmes and Barksdale, 1969). The *tox* locus, which represents the structural gene for diphtheria toxin (Uchida *et al*., 1971), is present in the genomes of several corynephages (Barksdale and Arden, 1974; Holmes and Barksdale, 1970). Expression of *tox* is not regulated coordinately with other corynephage genes (Gill *et al*., 1972; Matsuda and Barksdale, 1966), but production of diphtheria toxin is affected by the composition of the growth medium (Mueller, 1940; Mueller and Miller, 1941; Pappenheimer and Johnson, 1936). The component of the medium that has the largest and best studied effect on production of diphtheria toxin is iron.

Many bacterial pathogens respond to the severely limited availability of free iron in the host by expressing virulence factors (including toxins) (Cornelissen, 2003; Litwin and Calderwood, 1993; Rodriguez and Smith, 2003). Production of diphtheria toxin by toxinogenic *C. diphtheriae* is stringently repressed by high concentrations of iron in the extracellular environment *in vivo* and *in vitro*. An *in vitro* system provided the first evidence that a *C. diphtheriae* encoded product was responsible for this repression. In a transcription/translation system from *E. coli*, DNA from a $tox^+$ corynephage directed the synthesis of diphtheria toxin as well as other phage encoded proteins. Addition of *C. diphtheriae* extracts to this *in vitro* system resulted in inhibition of diphtheria toxin production, but it did not affect synthesis of the other phage proteins (Murphy *et al*., 1974). Several mutants of the corynephage were isolated that interfered with the ability of iron to repress diphtheria toxin production (Murphy *et al*., 1976; Uchida *et al*., 1977; Welkos and Holmes, 1981a; Welkos and Holmes, 1981b). In addition, mutations in the chromosome of *C. diphtheriae* that affected the ability of iron to repress diphtheria toxin productions were isolated (Kanei *et al*., 1977). These and other studies supported the hypothesis that a chromosomally encoded diphtheria toxin repressor existed and that it used iron as a co-repressor.

The cloning of the structural gene for diphtheria toxin *tox* occurred in the 1980s and led to the characterization of the contiguous upstream regulatory sequences (Greenfield *et al*., 1983; Kaczorek *et al*., 1983; Ratti *et al*., 1983). The promoter P-tox, at which transcription of *tox* initiates, was located and characterized shortly thereafter (Boyd and Murphy, 1988; Leong and Murphy, 1985). Finally in the early 1990s, the chromosomally encoded diphtheria toxin repressor DtxR was cloned (Boyd *et al*., 1990; Schmitt and Holmes, 1991). The physiologic role of DtxR is similar to that of the ferric uptake regulator Fur, which was previously found in several Gram-negative bacterial species. Fur and DtxR are now recognized as the prototypes for two different groups of iron-dependent bacterial repressor proteins.

The iron bound form of DtxR binds a DNA sequence that overlaps P-tox and prevents initiation of transcription. Thus, DtxR is an iron-dependent transcriptional repressor. The function and structure of DtxR have been extensively characterized (Feese *et al*., 2001; Holmes, 2000). In addition to *tox,* DtxR regulates several chromosomally encoded genes (Kunkle and Schmitt, 2003; Lee *et al*., 1997; Qian *et al*., 2002; Schmitt and Holmes, 1991; Schmitt and Holmes, 1994). Many DtxR regulated genes encode products that are involved in iron utilization and acquisition. Analysis of the DNA sequences bound by DtxR at different promoters has identified a 19 basepair consensus palindrome at the core of the DtxR binding site (Schmitt and Holmes, 1994; Tao and Murphy, 1994). Structurally, DtxR binds DNA as a dimer, and each DtxR monomer contains two metal binding sites. There are several specific contact sites between the DtxR dimer and the DNA (Chen *et al*., 2000; Pohl *et al*., 1999; White *et al*., 1998; Wisedchaisri *et al*., 2004).

Although $Fe^{2+}$ is the primary metal ion that acts as co-repressor *in vivo*, DtxR is capable of binding several divalent metals *in vitro*, including cadmium, cobalt, manganese, nickel, and zinc (Schmitt and Holmes, 1993; Schmitt *et al*., 1992; Tao *et al*., 1992; Tao and Murphy, 1992). DtxR-like regulator proteins have been identified in a wide variety of bacterial species where

they regulate gene expression in response to divalent metals (Feese *et al*., 2001; Guedon *et al*., 2003; Oram *et al*., 2004; Rodriguez and Smith, 2003). Interestingly, IdeR, the DtxR homologue in Mycobacterial species, has been shown to act as both a repressor and as an activator of transcription (Rodriguez and Smith, 2003). The characterization of the roles of DtxR-like regulators in virulence of bacterial pathogens, including Mycobacterial species, is of interest to a wide variety of researchers concerned with combating infectious disease.

## STRUCTURE OF DIPHTHERIA TOXIN

Diphtheria toxin is synthesized and, following removal of a signal sequence, is secreted by *C. diphtheriae* as a single polypeptide (Figure 13.1). The mature secreted toxin is 535 amino acid residues in length and has a molecular mass of approximately 58 kDa. The secreted holotoxin is a proenzyme that must be both cleaved and reduced in order for toxicity to be expressed. The holotoxin contains four cysteines that participate in two disulfide bonds: the first between Cys186 and Cys201 and the second between Cys461 and Cys471. *In vivo*, the proenzyme is cleaved, after binding its cellular receptor, by members of the furin family of cell-associated proteases (Gordon *et al*., 1995), and *in vitro* treatment of toxin with typsin and reduction of the "nicked" toxin produces two polypeptide fragments designated A and B. The amino terminal fragment A (molecular mass, about 21 kDa) is the catalytically active portion of toxin that mediates the intracellular action. The carboxyl terminal fragment B (molecular mass, about 37 kDa) contains the receptor binding and translocation portions of the toxin. Diphtheria toxin was the first described example of an A-B toxin, in which the catalytic and receptor-binding functions are positioned on separate polypeptides or on separate domains within a protein.

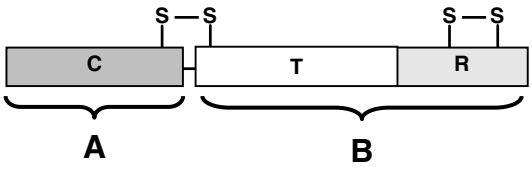



**FIGURE 13.1** Secondary structure of diphtheria toxin. The A and B fragments are labeled. The amino terminal catalytic domain (C) is shown in dark gray, the translocation domain (T) is shown in white, and the carboxyl terminal receptor binding (R) domain is shown in light gray. The Cys to Cys disulfide bonds are shown as S-S.

The first crystal structure of diphtheria toxin was reported in 1992 (Choe *et al*., 1992). The form crystallized was a dimer that was generated by freezing the toxin under certain conditions. The dimer is non-toxic but dissociates slowly at neutral pH to yield a toxic monomer. The structure of monomeric diphtheria toxin was solved shortly thereafter and revealed that the configurations of the individual domains were virtually identical to their counterparts in the dimer structure (Bennett and Eisenberg, 1994). Diphtheria toxin has three discrete domains C, T, and R, corresponding to the three major functions of the toxin: catalysis, translocation, and receptor-binding, respectively. The C domain corresponds to the A fragment and the T and R domains are contained within the B fragment.

The catalytic C domain of diphtheria toxin includes eight β strands and seven α helices, and the active site of the enzyme is formed by an α helix bent over a β strand. The catalytic activity of the C domain transfers the adenosine diphosphate ribose (ADP-ribose) moiety from nicotinamide adenine dinucleotide (NAD) to a modified hisitidine residue (diphthamide) in elongation factor 2 (EF-2), thereby inactivating protein synthesis in intoxicated cells (Collier, 1967; Honjo *et al*., 1968). The C domain binds NAD *in vivo*, but the first structures of diphtheria toxin included ApUp, an endogenous dinucleotide that is found in many toxin preparations occupying the active site cleft of the C domain. ApUp binds with high affinity and apparently acts as an NAD analog. Within the active site cavity, Tyr54 and Tyr65 are parallel with just enough space between their aromatic rings to allow entrance of the nicotinamide ring of NAD. His21 and Glu148, which are required for catalysis, are positioned on either side of the binding cavity. In addition, the crystal structure of dimeric diphtheria toxin in complex with NAD was solved in 1996, making it the first crystal structure of an ADP-ribosyltransferase in complex with NAD. In this structure, the carboxylate group of Glu148 was in proximity to the scissile, N-glycosidic bond of NAD (Bell and Eisenberg, 1996; Bell *et al*., 1997). This arrangement is consistent with the known requirement of Glu148 for ADP-ribosylation by diphtheria toxin. The structure of the active site appears to be conserved in all ADP-ribosylating enzymes (Domenighini *et al*., 1991, 1994).

The structure of the T domain of diphtheria toxin is organized in three layers of α helices. The outer layer contains three helices that are rich in charged residues and help maintain the T domain and diphtheria toxin in a soluble form at neutral pH. The middle layer contains three helices that form a hydrophobic shell, and the internal layer includes hydrophobic and amphipathic helices. At neutral pH, a central

hydrophobic helical hairpin is hidden from the solvent by the outer helices, and acidic pH induces a conformational change that results in a molten state with exposure of the hydrophobic parts of the central layer of helices, promoting interaction with membranes (Zhan *et al*., 1994). A Glu349Lys mutation, which blocks translocation, lies in an interhelix loop between the helices in that innermost helical hairpin. A positively charged Lys at this position would be expected to block insertion of this hairpin into the membrane, supporting a model in which the innermost helical hairpin represents a membrane-insertion motif that functions in translocation. Several recent results support this model (D'Silva and Lala, 2000; Kachel *et al*., 1998; Oh *et al*., 1999b; Ren *et al*., 1999b).

The R domain of diphtheria toxin is a globular domain that consists exclusively of β-sheets. The domain includes two β-sheets, each consisting of four β-strands. There is some evidence that the R domain may interact with membranes during its translocation (mediated by the T domain). Portions of the R domain are protected by membranes from external proteases, and the structure of the domain changes during interaction with membranes (Quertenmont *et al*., 1999). Within the R domain, Lys516 and Phe530 have a role in recognition of the receptor on the target cell (Shen *et al*., 1994). Overall, the structure of the diphtheria toxin R domain has similarities to the variable domain of immunoglobulins.

## DIPHTHERIA TOXIN RECEPTOR

The first step in intoxication of eukaryotic cells by diphtheria toxin is the binding of the toxin to a specific cellular receptor. The gene that encodes the receptor for diphtheria toxin was first cloned as a cDNA from toxin sensitive monkey Vero cells and was able to confer toxin sensitivity to the normally toxin-resistant mouse L-M(TK-) cell line (Naglich *et al*., 1992a). The cDNA encoded a protein with a predicted amino acid sequence that was identical to that of the heparin-binding epidermal growth factor precursor (pro-HB-EGF). The heparin-binding epidermal growth factor (HB-EGF) is a member of the EGF family, and it is derived through a proteolytic cleavage of the surface-exposed, membrane-bound pro-HB-EGF (Higashiyama *et al*., 1991). Although pro-HB-EGF is cleaved to release soluble HB-EGF, a significant amount of pro-HB-EGF remains on the cell surface where it functions as a growth factor (Higashiyama *et al*., 1995) and serves as a receptor for diphtheria toxin (Iwamoto *et al*., 1994; Naglich *et al*., 1992a).

On the cell surface, pro-HB-EGF forms a complex with several other membrane proteins, including heparin sulfate proteoglycans and CD9 (Iwamoto *et al*., 1991; Shishido *et al*., 1995), both of which are known to enhance the ability of the receptor to bind diphtheria toxin. The effects of heparin-like molecules, including heparin sulfate proteoglycans, and CD9 on the binding of diphtheria toxin are additive, suggesting that they influence binding by different mechanisms. Heparin-like molecules bind to the heparin-binding domain of pro-HB-EGF, causing a change in the conformation of the domain that increases the affinity of the receptor for diphtheria toxin (Shishido *et al*., 1995). The precise mechanism by which CD9 promotes pro-HB-EGF binding to diphtheria toxin has not been determined, but direct protein to protein contact between the two molecules is required and regions of CD9 essential for this contact have been identified (Hasuwa *et al*., 2001; Nakamura *et al*., 2000; Ryu *et al*., 2000).

Most mammalian species are sensitive to the effects of diphtheria toxin, but mice and rats are highly resistant to diphtheria toxin. The resistance of these species to the action of diphtheria toxin is caused by the lack of a functional receptor on cell surfaces (Cha *et al*., 1998). Recently, several authors have used the natural resistance of mice to diphtheria toxin as a tool to study the roles of specific cell types in development and disease. The first transgenic mice expressing a functional receptor for diphtheria toxin were described in 2001 (Saito *et al*., 2001). These mice expressed human pro-HB-EGF under control of a hepatocyte-specific promoter. The injection of diphtheria toxin resulted in rapid and severe hepatitis, thus providing a useful model for disease. This hepatitis model was the first description of "toxin receptor-mediated cell knockout" in which the expression of the diphtheria toxin receptor permits the expressing cell type to be specifically eliminated through the administration of diphtheria toxin without affecting other cells. Since 2001 other lines of "toxin receptor-mediated cell knockout" mice have been engineered and used successfully to study the functions of cell types other than liver cells, including dendritic cells and cardiomyocytes (Akazawa *et al*., 2004; Jung *et al*., 2002). This technology provides a powerful tool for researchers wanting to precisely and specifically eliminate any mouse cell type that can be engineered to express human pro-HB-EGF, at any time during pre- or postnatal development, through the administration of diphtheria toxin.

In addition to transgenic mice expressing the diphtheria toxin receptor in specific cell types, a line of mice expressing the receptor in most cell types has been engineered (Cha *et al*., 2003). In these mice the expression of human pro-HB-EGF is under control of a

cytomegalovirus promoter that is activated postnatally, since embryonic overexpression of pro-HB-EGF in all mouse cells proved to be fatal. The transgenic mice expressing pro-HB-EGF are sensitive to diphtheria toxin at a concentration of 0.1 μg per kg, which is similar to that of sensitive species such as guinea pigs (Collier, 1975). These mice were used to test the ability of a soluble receptor-based, toxin-binding peptide (Cha *et al*., 2002) to protect against the action of diphtheria toxin, and they can be used to test any potential antidote for diphtheria toxin.

## INTERNALIZATION AND TRANSLOCATION OF DIPHTHERIA TOXIN

After binding to pro-HB-EGF on the surface of a susceptible cell, diphtheria toxin is internalized in a process termed *receptor-mediated endocytosis*. Specifically, diphtheria toxin enters cells by clathrin-mediated endocytosis (Moya *et al*., 1985; Simpson *et al*., 1998), and it has been visualized in clathrin-coated pits (Morris *et al*., 1985). In confirmation of this mechanism of entry, lowering the intracellular potassium level, which inhibits pit formation and clathrin-mediated endocytosis, protects cells from the effects of diphtheria toxin (Moya *et al*., 1985; Sandvig *et al*., 1985). In addition, the cytoplasmic domain of pro-HB-EGF has a tyrosine-containing sequence (Naglich *et al*., 1992b), which may serve to anchor the receptor in clathrin-coated pits and ensure that diphtheria toxin enters the cell via clathrin-dependent endocytosis.

Although the precise molecular mechanism by which diphtheria toxin translocates across the endosomal membrane and is released into the cytoplasm is an area of active investigation, several steps in the process have been characterized. The translocation of diphtheria toxin across membranes can be observed in cell-free systems, suggesting that the toxin alone has the ability to penetrate and transverse lipid bilayers (Jiang *et al*., 1991; Oh *et al*., 1999a; Umata and Mekada, 1998). In addition, it has been demonstrated that translocation of diphtheria toxin from the lumen of early endosomes to the external milieu requires the addition of a cytosolic translocation factor complex that includes heat shock protein 90 and thioredocin reductase (Ratts *et al*., 2003). The proposed role of the cytosolic translocation factor complex is to aid in release and refolding of the C domain of diphtheria toxin after translocation from early endosomes. Several lines of evidence support the theory that the catalytic C domain (in the A fragment) of diphtheria toxin is denatured prior to translocation (Donovan *et al*., 1981; Falnes *et al*., 1994; Kagan *et al*., 1981; Wiedlocha *et al*., 1992). Interestingly, a direct interaction between the C domain and lipid membranes has recently been demonstrated (Wolff *et al*., 2004).

The T domain of diphtheria toxin has a critical role in translocation of the toxin. It is a largely α helical domain, and it has a hydrophobic carboxyl terminal portion that has the ability to insert into and form pores within both biological and model membranes under conditions of low pH (Kagan *et al*., 1981; Papini *et al*., 1988; Sandvig and Olsnes, 1988; Sharpe and London, 1999). The T domain of diphtheria toxin can also assist the translocation of some heterologous proteins across lipid bilayers at low pH (Ren *et al*., 1999a). Following endocytosis, the low pH of the endosomes induces a conformational change that results in diphtheria toxin dissociating from pro-HB-EGF (Brooke *et al*., 1998) and interacting with the endosomal membrane (Sandvig and Olsnes, 1980). The interaction of the T domain with lipid membranes is an area of active research as a model for trafficking across membranes. Hydrophobic helices 8 and 9 of the T domain form a transmembrane helical hairpin (Moskaug *et al*., 1991; Oh *et al*., 1999b; Quertenmont *et al*., 1996), and a second transmembrane hairpin is predicted to be formed by helices 5, 6, and 7, although there is less supporting evidence for this structure than that for the transmembrane helical hairpin formed by helices 8 and 9. Recent evidence suggests that helices 5, 6, and 7 may form stable but nonclassical inserted segments in lipid bilayers (Rosconi *et al*., 2004). Further details of toxin translocation and intracellular trafficking can be found in chapters 7 and 8.

## ACTIVITY OF DIPHTHERIA TOXIN AND INTRACELLULAR TARGET

Diphtheria toxin kills cells by inhibiting eukaryotic protein synthesis, and its mechanism of action has been extensively characterized. This potent toxin inactivates elongation factor (EF-2) required for protein synthesis (Collier, 1967). Specifically, diphtheria toxin transfers the ADP-ribose moiety of NAD to EF-2 (first demonstrated using radiolabeled NAD) (Honjo *et al*., 1968). Diphtheria toxin was the first member to be identified of a group of bacterial protein toxins that act by ADP-ribosylation of a target protein. The ADP-ribosylation activity of diphtheria toxin is determined completely by the A fragment, and no portion of the B fragment is required for catalytic activity.

The intracellular toxicity of the A fragment of diphtheria toxin is preserved even when it is produced

independently from the B fragment. Therefore, tissue-specific intracellular expression of the diphtheria toxin A fragment can be used to kill particular cell types. This method has been used extensively to study eukaryotic development. Briefly, the coding sequence for the A fragment of diphtheria toxin is placed under the transcriptional control of a tissue-specific eukaryotic promoter and the resulting promoter-gene fusion is integrated into embryonic cells. As the organism develops, the tissue specific promoter is activated, and expression of the diphtheria toxin A fragment results in death of all cells in which the tissue-specific promoter is active. Using this method, the developmental roles of several cell types have been determined, including those involved in development of the eye, pancreas, kidney, and roof plate in mice (Breitman *et al*., 1987; Lee *et al*., 2000; Palmiter *et al*., 1987; Pentz *et al*., 2004). This method is applicable to a wide variety of plants, animals, and insects, including *Drosophila*, rainbow trout, and *Arabidopsis* (Han *et al*., 2000; Kalb *et al*., 1993; Tsugeki and Fedoroff, 1999; Uzbekova *et al*., 2003). In some cases, production of wild-type A fragment results in lethality early in development so mutant forms of the A fragment that are less active, and therefore less toxic, than wild-type have been used to kill cells slower and later in development. Characterization of the active site of the A fragment provided the basis for the development of less toxic forms of diphtheria toxin.

The amino acids that make up the active site of the A fragment have been characterized using several methods. There is only one amino acid residue, Glu148, that is completely conserved within the family of ADP-ribosyltransferase toxins. Glu148 is essential for the ADP ribosylation activity of the A fragement, and it is positioned near the NAD binding pocket in the crystal structure (Carroll and Collier, 1984; Choe *et al*., 1992; Tweten *et al*., 1985). His21 is also required for catalytic activity, and it is important for NAD binding (Papini *et al*., 1990; Papini *et al*., 1989). Another amino acid residue, Tyr65, was initially identified by biochemical studies as important for binding NAD (Papini *et al*., 1991), and site-directed mutations have confirmed its importance in catalysis (Blanke *et al*., 1994). In addition, Tyr50 is required for full enzymatic activity. A substitution of Try50 with Ala drastically decreased the enzymatic activity (105-fold), but a substitution of Phe had a very small effect. This suggests a role for an aromatic residue at position 50 (Wilson *et al*., 1994).

The intracellular target of ADP ribosylation by diphtheria toxin, EF-2, mediates the translocation step in peptide chain elongation by promoting transfer of peptidyl tRNA from the A to the P site of the ribosome. The amino acid residue of EF-2 that is ADP-ribosylated by diphtheria toxin is a posttranslationally modified histidine called *diphthamide*. The diphthamide residue is unique to EF-2 from eukaryotes and Archea and is not found in any other protein (Bodley *et al*., 1984; Collier, 2001). Synthesis of diphthamide is a complex process that requires the participation of at least seven different gene products (Liu and Leppla, 2003; Liu *et al*., 2004; Mattheakis *et al*., 1993; Schultz *et al*., 1998). The retention of the diphthamide biosynthesis pathway by eukaryotes and Archea implies a significant function of diphthamide apart from its inactivation by diphtheria toxin, although mutant cell lines that cannot produce diphthamide remain viable and are resistant to diphtheria toxin (Phan *et al*., 1993). It has been suggested that ADP ribosylation of diphthamide in EF-2 may occur as a regulatory event in normal cellular physiology (Fendrick *et al*., 1992), but evidence for the precise physiological role of diphthamide in eukaryotes remains elusive.

## DIPHTHERIA TOXIN AS A VACCINE COMPONENT

In addition to the use of the diphtheria toxid for universal immunization against diphtheria, non-toxic mutants of diphtheria toxin have been used as a component of vaccines designed to elicit immune responses to antigens from a variety of bacterial pathogens. The non-toxic diphtheria toxin molecules increase both the cell-mediated and humoral immune responses to some specific antigens that elicit poor immune responses when administered alone. The most common non-toxic diphtheria toxin mutants used to enhance immune responses are CRM197 (Uchida *et al*., 1973), which contains a single amino acid change in the catalytic portion of the molecule (Giannini *et al*., 1984), and CRM9, which has a single amino acid change in the B fragment that lowers its affinity for the receptor (Hu and Holmes, 1987). CRM9 conjugate vaccines are being developed against diseases caused by *Shigella* and *Moraxella* species (Jiao *et al*., 2002; Passwell *et al*., 2003). CRM197 conjugate vaccines directed against many different bacterial antigens including *Haemophilus influenzae* type b capsular antigen, as well as meningococcal, pneumococcal, mycobacterial, and streptococcal antigens, have already been proven to be safe and effective (Baker *et al*., 2004; Baraldo *et al*., 2004; Klugman *et al*., 2003; Mawas *et al*., 2004; Mazzantini *et al*., 2004). In addition, new vaccines based on CRM197 and directed against diphtheria are also being developed (McNeela *et al*., 2004; Rydell and Sjoholm, 2004). Further information about the ability of bacterial toxins to affect the immune response can be found in Section V, Chapters 54 and 61.

## POTENTIAL MEDICAL USES OF THE DIPHTHERIA TOXIN R DOMAIN AND THE CELLULAR RECEPTOR

Patients with diphtheria are treated with equine diphtheria antitoxin, which neutralizes unbound diphtheria toxin. This treatment can result in serum sickness, an immune complex disease, so a better treatment for diphtheria patients would be desirable. One approach to developing a new treatment is based on preventing diphtheria toxin binding to its cognate receptor pro-HB-EGF. While the soluble form of HB-EGF will bind diphtheria toxin and block its binding to pro-HB-EGF (Hooper and Eidels, 1995), soluble HB-EGF is likely to have side effects based on its growth factor activity. The careful characterization of the region of pro-HB-EGF required for binding of diphtheria toxin has laid the groundwork for the development of a receptor-blocking–based antidote for diphtheria. The region of pro-HB-EGF required for binding of toxin was identified by expressing chimeric fusions between the murine pro-HB-EGF receptor, which does not bind diphtheria toxin, and either human or monkey receptors, both of which bind diphtheria toxin (Hooper and Eidels, 1995; Hooper and Eidels, 1996; Mitamura *et al*., 1995). These studies localized the toxin binding region to an extracellular portion of the protein within the EGF domain. In addition, several specific amino acid residues, including Phe115, Leu127; His135, and Glu141, were identified as being critical for toxin binding (Cha *et al*., 1998; Hooper and Eidels, 1996; Mitamura *et al*., 1997). Recently, a recombinant truncated form of HB-EGF that blocks binding of diphtheria toxin but has 100-fold lower mitogenic activity than full-length HB-EGF was described (Cha *et al*., 2002). Further refinement of this receptor-blocking recombinant protein may lead to a new treatment for diphtheria without the possible side effects of serum sickness.

Since the catalytic activity of fragment A is not required for binding of diphtheria toxin to cognate receptor pro-HB-EGF, some non-toxic mutants of diphtheria toxin can bind the receptor with an affinity equal to that of wild-type toxin. The non-toxic mutant form of diphtheria toxin CRM197 (Uchida *et al*., 1973) has been studied extensively for its ability to act as a carrier in conjugate vaccines (discussed previously), and it is now being used to block the signaling activity of the receptor. The ability of the non-toxic CRM197 to bind the pro-HB-EGF receptor and inhibit this receptor's mitogenic activity (Mitamura *et al*., 1995) has provided the basis for the development of new methods to treat some forms of cancer. In some cancers, including ovarian cancer, pro-HB-EGF is overexpressed and shed. In such cancers, blocking the ability of pro-HB-EGF to signal using CRM197 has shown some promise as a therapy (Buzzi *et al*., 2004; Miyamoto *et al*., 2004). This area of research is likely to grow as the precise effects of pro-HB-EGF up-regulation on cancer development and spread are elucidated.

## USES OF THE DIPHTHERIA TOXIN C AND T DOMAINS IN TREATMENT OF DISEASE

The high potency of diphtheria toxin is advantageous to researchers who want to eliminate specific cell types efficiently. If the receptor-binding R domain of diphtheria toxin is replaced with another protein domain capable of recognizing specific cell surface expressed molecules, the catalytic C domain and the translocation T domain can be delivered specifically to cells that display such molecules. There are currently two types of molecules that have been fused to the C and T domains of diphtheria toxin with the goal of facilitating the delivery of the catalytically active portion of the toxin to specific cell types.

First, the C and T domains of diphtheria toxin have been fused to antibodies that recognize cell surface epitopes, and such constructs are called *immunotoxins*. Recombinant immunotoxins are antibody-toxin chimeric molecules that kill cells by binding to a surface antigen and delivering the toxin portion to the cell cytosol. Diphtheria toxin based immunotoxins are in development for use against several forms of leukemia and other cancers (FitzGerald *et al*., 2004; Frankel *et al*., 2001). Second, the C and T domains of diphtheria toxin have been fused to growth factors and cytokines that bind to specific cell surface receptors. The fusion proteins have included granulocyte macrophage-colony stimulating factor and IL-3, whose receptors are overexpressed on many acute myeloid leukemias, and IL-2, whose receptor is expressed by many lymphoma and leukemia cells (Hogge *et al*., 2004; Walker and Dang, 2004). The use of protein toxins in tumor therapy is discussed in more detail in Section V, Chapter 60.

## CONCLUSION

Years of studying the structure and function of diphtheria toxin and its receptor have made diphtheria toxin one of the best characterized bacterial protein toxins. The modular A-B structure of the toxin is amenable to manipulations that separate the catalytic domain from the translocation and receptor binding portions. In addition, the mechanism of ADP-ribosylation used by diphtheria toxin to modify its cellular target has provided a model for the action of other

ADP-ribosylating enzymes. All of this information is now being used for applications in a wide variety of biological science areas, including eukaryotic cell biology, developmental biology, signal transduction, protein translation, and cancer research.

Even though diphtheria toxin is well characterized, there are several important aspects of its action that merit further investigation. Two of these are the mechanism of translocation of the toxin across cellular membranes and the precise sequence of events that lead from toxic effects on particular cells, tissues, and organs to the death of the affected human or animal. In addition, the physiologic role of the target of diphtheria toxin, the diphthamide residue of EF-2, remains to be elucidated. The structure and function of diphtheria toxin, and uses of the toxin as a molecular tool to examine other biological problems, continue to be vigorous and productive areas of research more than 100 years after the discovery of diphtheria toxin.

## ACKNOWLEDGMENTS

D. M. O. was supported in part by postdoctoral training grant number T32 AI007537, and work in the laboratory of R. K. H. was supported in part by grant number AI 14107, both from the National Institute of Allergy and Infectious Disease, National Institutes of Health.

## REFERENCES

Akazawa, H., Komazaki, S., Shimomura, H., Terasaki, F., Zou, Y., Takano, H., Nagai, T. and Komuro, I. (2004). Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure. *J. Biol. Chem*. **279**, 41095–41103.

Baker, C.J., Paoletti, L.C., Rench, M. A., Guttormsen, H.K., Edwards, M.S. and Kasper, D.L. (2004). Immune response of healthy women to two different group B streptococcal type V capsular polysaccharide-protein conjugate vaccines. *J. Infect. Dis*. **189**, 1103–1112.

Baraldo, K., Mori, E., Bartoloni, A., Petracca, R., Giannozzi, A., Norelli, F., Rappuoli, R., Grandi, G. and Del Giudice, G. (2004). N19 polyepitope as a carrier for enhanced immunogenicity and protective efficacy of meningococcal conjugate vaccines. *Infect. Immun*. **72**, 4884–4887.

Barksdale, L. and Arden, S.B. (1974). Persisting bacteriophage infections, lysogeny, and phage conversions. *Annu. Rev. Microbiol*. **28**, 265–299.

Barksdale, L.W. and Pappenheimer, A.M.J. (1954). Phage-host relationships in nontoxigenic and toxigenic diphtheria bacilli. *J. Bacteriol*. **67**, 220–232.

Bennett, M.J. and Eisenberg, D. (1994). Refined structure of monomeric diphtheria toxin at 2.3 A resolution. *Protein Sci*. **3**, 1464–1475.

Blanke, S. R., Huang, K. and Collier, R. J. (1994). Active site mutations of diphtheria toxin: role of tyrosine-65 in NAD binding and ADP-ribosylation. *Biochemistry* **33**, 15494–15500.

Bodley, J.W., Dunlop, P.C. and VanNess, B.G. (1984). Diphthamide in elongation factor 2: ADP-ribosylation, purification, and properties. *Methods Enzymol*. **106**, 378–387.

Boyd, J. and Murphy, J.R. (1988). Analysis of the diphtheria tox promoter by site-directed mutagenesis. *J. Bacteriol*. **170**, 5949–5952.

Boyd, J., Oza, M.N. and Murphy, J.R. (1990). Molecular cloning and DNA sequence analysis of a diphtheria tox iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae. Proc. Natl. Acad. Sci. USA* **87**, 5968–5972.

Breitman, M.L., Clapoff, S., Rossant, J., Tsui, L.C., Glode, L.M., Maxwell, I.H. and Bernstein, A. (1987). Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. *Science* **238**, 1563–1565.

Brooke, J.S., Cha, J.H. and Eidels, L. (1998). Diphtheria toxin: receptor interaction: association, dissociation, and effect of pH. *Biochem. Biophys. Res. Commun*. **248**, 297–302.

Buzzi, S., Rubboli, D., Buzzi, G., Buzzi, A.M., Morisi, C. and Pironi, F. (2004). CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients. *Cancer Immunol. Immunother*. **53**, 1041–1048.

Carroll, S.F. and Collier, R.J. (1984). NAD binding site of diphtheria toxin: identification of a residue within the nicotinamide subsite by photochemical modification with NAD. *Proc. Natl. Acad. Sci. USA* **81**, 3307–3311.

Cha, J.H., Brooke, J.S., Chang, M.Y. and Eidels, L. (2002). Receptor-based antidote for diphtheria. *Infect. Immun*. **70**, 2344–2350.

Cha, J.H., Brooke, J. S. and Eidels, L. (1998). Toxin binding site of the diphtheria toxin receptor: loss and gain of diphtheria toxin binding of monkey and mouse heparin-binding, epidermal growth factor-like growth factor precursors by reciprocal site-directed mutagenesis. *Mol. Microbiol*. **29**, 1275–1284.

Cha, J.H., Chang, M.Y., Richardson, J.A. and Eidels, L. (2003). Transgenic mice expressing the diphtheria toxin receptor are sensitive to the toxin. *Mol. Microbiol*. **49**, 235–240.

Chen, C.S., White, A., Love, J., Murphy, J.R. and Ringe, D. (2000). Methyl groups of thymine bases are important for nucleic acid recognition by DtxR. *Biochemistry* **39**, 10397–10407.

Chen, R.T., Broome, C.V., Weinstein, R.A., Weaver, R. and Tsai, T.F. (1985). Diphtheria in the United States, 1971-81. *Am. J. Public Health* **75**, 1393–1397.

Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M., Kantardjieff, K.A., Collier, R.J. and Eisenberg, D. (1992). The crystal structure of diphtheria toxin. *Nature* **357**, 216–222.

Collier, R.J. (1967). Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. *J. Mol. Biol*. **25**, 83–98.

Collier, R.J. (1975). Diphtheria toxin: mode of action and structure. *Bacteriol. Rev*. **39**, 54–85.

Collier, R.J. (2001). Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. *Toxicon*. **39**, 1793–1803.

Cornelissen, C.N. (2003). Transferrin-iron uptake by Gram-negative bacteria. *Front. Biosci*. **8**, d836–847.

Dittmann, S., Wharton, M., Vitek, C., Ciotti, M., Galazka, A., Guichard, S., Hardy, I., Kartoglu, U., Koyama, S., Kreysler, J. *et al*. (2000). Successful control of epidemic diphtheria in the states of the former Union of Soviet Socialist Republic: lessons learned. *J. Infect. Dis*. **181**, S10–22.

Donovan, J.J., Simon, M.I., Draper, R.K. and Montal, M. (1981). Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. USA* **78**, 172–176.

D'Silva, P.R. and Lala, A.K. (2000). Organization of diphtheria toxin in membranes. A hydrophobic photolabeling study. *J. Biol. Chem*. **275**, 11771–11777.

Falnes, P.O., Choe, S., Madshus, I.H., Wilson, B.A. and Olsnes, S. (1994). Inhibition of membrane translocation of diphtheria toxin

A-fragment by internal disulfide bridges. *J. Biol. Chem*. **269**, 8402–8407.

Feese, M.D., Pohl, E., Holmes, R.K. and Hol, W.G.J. (2001). Iron-dependent regulators. In: *Handbook of Metalloproteins* (eds. A. Messerschmidt, R. Huber, R. Poulos, and K. Wieghardt), pp. 850–863. John Wiley & Sons, Ltd, Chichester.

Fendrick, J.L., Iglewski, W.J., Moehring, J.M. and Moehring, T. J. (1992). Characterization of the endogenous ADP-ribosylation of wild-type and mutant elongation factor 2 in eukaryotic cells. *Eur. J. Biochem*. **205**, 25–31.

FitzGerald, D.J., Kreitman, R., Wilson, W., Squires, D. and Pastan, I. (2004). Recombinant immunotoxins for treating cancer. *Int. J. Med. Microbiol*. **293**, 577–582.

Frankel, A.E., Powell, B.L., Vallera, D.A. and Neville, D.M., Jr. (2001). Chimeric fusion proteins—diphtheria toxin-based. *Curr. Opin. Investig. Drugs* **2**, 1294–1301.

Freeman, V.J. (1951). Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae. J. Bacteriol*. **61**, 675–688.

Giannini, G., Rappuoli, R. and Ratti, G. (1984). The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res*. **12**, 4063–4069.

Gilbert, L. (1997). Infections with *Corynebacterium diphtheriae*—changing epidemiology and clinical manifestations. Report of the third international meeting of the European Laboratory Working Group on Diphtheria (ELWGD), Institute Pasteur, Paris 7–8 June 1996. *Commun. Dis. Intell*. **21**, 161–164.

Gill, D.M., Uchida, T. and Singer, R. A. (1972). Expression of diphtheria toxin genes carried by integrated and nonintegrated phage beta. *Virology* **50**, 664–668.

Gordon, V.M., Klimpel, K.R., Arora, N., Henderson, M.A. and Leppla, S. H. (1995). Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. *Infect. Immun*. **63**, 82–87.

Greenfield, L., Bjorn, M.J., Horn, G., Fong, D., Buck, G.A., Collier, R. J. and Kaplan, D.A. (1983). Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage beta. *Proc. Natl. Acad. Sci. USA* **80**, 6853–6857.

Groman, N.B. (1953). Evidence for the induced nature of the change from non-toxigenicity to toxigenicity in *Corynebacterium diphtheriae* as a result of exposre to specific bacteriophage. *J. Bacteriol*. **66**, 184–191.

Guedon, E., Moore, C.M., Que, Q., Wang, T., Ye, R.W. and Helmann, J. D. (2003). The global transcriptional response of *Bacillus subtilis* to manganese involves the MntR, Fur, TnrA, and sigmaB regulons. *Mol. Microbiol*. **49**, 1477–1491.

Han, D.D., Stein, D. and Stevens, L.M. (2000). Investigating the function of follicular subpopulations during Drosophila oogenesis through hormone-dependent enhancer-targeted cell ablation. *Development* **127**, 573–583.

Harnisch, J.P., Tronca, E., Nolan, C.M., Turck, M. and Holmes, K.K. (1989). Diphtheria among alcoholic urban adults. A decade of experience in Seattle. *Ann. Intern. Med*. **111**, 71–82.

Hasuwa, H., Shishido, Y., Yamazaki, A., Kobayashi, T., Yu, X. and Mekada, E. (2001). CD9 amino acids critical for up-regulation of diphtheria toxin binding. *Biochem. Biophys. Res. Commun*. **289**, 782–790.

Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. and Klagsbrun, M. (1991). A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936–939.

Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J. Cell. Biol*. **128**, 929–938.

Hogge, D.E., Feuring-Bus ke, M., Gerhard, B. and Frankel, A.E. (2004). The efficacy of diphtheria-growth factor fusion proteins is enhanced by co-administration of cytosine arabinoside in an immunodeficient mouse model of human acute myeloid leukemia. *Leuk. Res*. **28**, 1221–1226.

Holmes, R.K. (2000). Biology and molecular epidemiology of diphtheria toxin and the *tox* gene. *Journal of Infectious Disease* **181**, S156–167.

Holmes, R.K. (2005). Diphtheria and other corynebacterial infections. In: *Harrison's Principals of Internal Medicine*, (eds. D. L. Kasper, E. Braunwald, A. S. Fauci, S. L. Hauser, D. L. Longo, J. L. Jameson, and K. J. Isselbacher), pp. 832–837 McGraw-Hill, New York.

Holmes, R.K. and Barksdale, L. (1969). Genetic analysis of tox+ and tox– bacteriophages of *Corynebacterium diphtheriae. J. Virol*. **3**, 586–598.

Holmes, R.K. and Barksdale, L. (1970). Comparative studies with tox plus and tox minus corynebacteriophages. *J. Virol*. **5**, 783–784.

Honjo, T., Nishizuka, Y. and Hayaishi, O. (1968). Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem*. **243**, 3553–3555.

Hooper, K.P. and Eidels, L. (1995). Localization of a critical diphtheria toxin-binding domain to the C-terminus of the mature heparin-binding EGF-like growth factor region of the diphtheria toxin receptor. *Biochem. Biophys. Res. Commun*. **206**, 710–717.

Hooper, K.P. and Eidels, L. (1996). Glutamic acid 141 of the diphtheria toxin receptor (HB-EGF precursor) is critical for toxin binding and toxin sensitivity. *Biochem. Biophys. Res. Commun*. **220**, 675–680.

Hu, V.W. and Holmes, R.K. (1987). Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behavior. *Biochim. Biophys. Acta*. **902**, 24–30.

Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1994). Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *Embo. J*. **13**, 2322–2330.

Iwamoto, R., Senoh, H., Okada, Y., Uchida, T. and Mekada, E. (1991). An antibody that inhibits the binding of diphtheria toxin to cells revealed the association of a 27-kDa membrane protein with the diphtheria toxin receptor. *J. Biol. Chem*. **266**, 20463–20469.

Jiang, J.X., Chung, L.A. and London, E. (1991). Self-translocation of diphtheria toxin across model membranes. *J. Biol. Chem*. **266**, 24003–24010.

Jiao, X., Hirano, T., Hou, Y. and Gu, X.X. (2002). Specific immune responses and enhancement of murine pulmonary clearance of *Moraxella catarrhalis* by intranasal immunization with a detoxified lipooligosaccharide conjugate vaccine. *Infect. Immun*. **70**, 5982–5989.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F. *et al.* (2002). *In vivo* depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* **17**, 211–220.

Kachel, K., Ren, J., Collier, R. J. and London, E. (1998). Identifying transmembrane states and defining the membrane insertion boundaries of hydrophobic helices in membrane-inserted diphtheria toxin T domain. *J. Biol. Chem*. **273**, 22950–22956.

Kaczorek, M., Delpeyroux, F., Chenciner, N., Streeck, R. E., Murphy, J. R., Boquet, P. and Tiollais, P. (1983). Nucleotide sequence and expression of the diphtheria tox228 gene in *Escherichia coli. Science* **221**, 855–858.

Kagan, B.L., Finkelstein, A. and Colombini, M. (1981). Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **78**, 4950–4954.

Kalb, J.M., DiBenedetto, A.J. and Wolfner, M.F. (1993). Probing the function of *Drosophila melanogaster* accessory glands by directed cell ablation. *Proc. Natl. Acad. Sci. USA* **90**, 8093–8097.

Kanei, C., Uchida, T. and Yoneda, M. (1977). Isolation from *Corynebacterium diphtheriae* C7(beta) of bacterial mutants that produce toxin in medium with excess iron. *Infect. Immun*. **18**, 203–209.

Klugman, K.P., Madhi, S.A., Huebner, R.E., Kohberger, R., Mbelle, N. and Pierce, N. (2003). A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N. Engl. J. Med*. **349**, 1341–1348.

Kunkle, C.A. and Schmitt, M.P. (2003). Analysis of the Corynebacterium diphtheriae DtxR regulon: identification of a putative siderophore synthesis and transport system that is similar to the Yersinia high-pathogenicity island-encoded yersiniabactin synthesis and uptake system. *J. Bacteriol*. **185**, 6826–6840.

Lee, J.H., Wang, T., Ault, K., Liu, J., Schmitt, M.P. and Holmes, R.K. (1997). Identification and characterization of three new promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *Infect. Immun*. **65**, 4273–4280.

Lee, K.J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734–740.

Lennox, E.S. and Kaplan, A.S. (1957). Action of diphtheria toxin on cells cultivated *in vitro. Proc. Soc. Exp. Biol. Med*. **95**, 700–702.

Leong, D. and Murphy, J.R. (1985). Characterization of the diphtheria *tox* transcript in *Corynebacterium diphtheriae* and *Escherichia coli. J. Bacteriol*. **163**, 1114–1119.

Litwin, C.M. and Calderwood, S.B. (1993). Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev*. **6**, 137–149.

Liu, S. and Leppla, S.H. (2003). Retroviral insertional mutagenesis identifies a small protein required for synthesis of diphthamide, the target of bacterial ADP-ribosylating toxins. *Mol. Cell*. **12**, 603–613.

Liu, S., Milne, G.T., Kuremsky, J.G., Fink, G.R. and Leppla, S.H. (2004). Identification of the proteins required for biosynthesis of diphthamide, the target of bacterial ADP-ribosylating toxins on translation elongation factor 2. *Mol. Cell. Biol*. **24**, 9487–9497.

Matsuda, M. and Barksdale, L. (1966). Phage-directed synthesis of diphtherial toxin in non-toxinogenic *Corynebacterium diphtheriae. Nature* **210**, 911–913.

Mattheakis, L.C., Sor, F. and Collier, R.J. (1993). Diphthamide synthesis in *Saccharomyces cerevisiae*: structure of the DPH2 gene. *Gene* **132**, 149–154.

Mawas, F., Peyre, M., Beignon, A.S., Frost, L., Del Giudice, G., Rappuoli, R., Muller, S., Sesardic, D. and Partidos, C. D. (2004). Successful induction of protective antibody responses against Haemophilus influenzae type b and diphtheria after transcutaneous immunization with the glycoconjugate polyribosyl ribitol phosphate-cross-reacting material 197 vaccine. *J. Infect. Dis*. **190**, 1177–1182.

Mazzantini, R.P., Miyaji, E.N., Dias, W.O., Sakauchi, D., Nascimento, A. L., Raw, I., Winter, N., Gicquel, B., Rappuoli, R. and Leite, L. C. (2004). Adjuvant activity of Mycobacterium bovis BCG expressing CRM197 on the immune response induced by BCG expressing tetanus toxin fragment C. *Vaccine* **22**, 740–746.

McNeela, E.A., Jabbal-Gill, I., Illum, L., Pizza, M., Rappuoli, R., Podda, A., Lewis, D.J. and Mills, K.H. (2004). Intranasal immunization with genetically detoxified diphtheria toxin induces T-cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan. *Vaccine* **22**, 909–914.

Mitamura, T., Higashiyama, S., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem*. **270**, 1015–1019.

Mitamura, T., Umata, T., Nakano, F., Shishido, Y., Toyoda, T., Itai, A., Kimura, H. and Mekada, E. (1997). Structure-function analysis of the diphtheria toxin receptor toxin binding site by site-directed mutagenesis. *J. Biol. Chem*. **272**, 27084–27090.

Miyamoto, S., Hirata, M., Yamazaki, A., Kageyama, T., Hasuwa, H., Mizushima, H., Tanaka, Y., Yagi, H., Sonoda, K., Kai, M., *et al*. (2004). Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. *Cancer Res*. **64**, 5720–5727.

Morris, R.E., Gerstein, A.S., Bonventre, P.F. and Saelinger, C.B. (1985). Receptor-mediated entry of diphtheria toxin into monkey kidney (Vero) cells: electron microscopic evaluation. *Infect. Immun*. **50**, 721–727.

Moskaug, J.O., Stenmark, H. and Olsnes, S. (1991). Insertion of diphtheria toxin B-fragment into the plasma membrane at low pH. Characterization and topology of inserted regions. *J. Biol. Chem*. **266**, 2652–2659.

Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. and Boquet, P. (1985). Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell. Biol*. **101**, 548–559.

Mueller, J.H. (1940). Nutrition of the diphtheria bacillus. *Bacteriol Rev*. **4**, 97–134.

Mueller, J.H. and Miller, P.A. (1941). Production of diphtheric toxin of high potency (100 Lf) on a reproducible medium. *J. Immunol*. **40**, 21–32.

Murphy, J.R., Pappenheimer, A.M. and de Borms, S.T. (1974). Synthesis of diphtheria tox-gene products in *Escherichia coli* extracts. *Proc. Natl. Acad. Sci. USA* **71**, 11–15.

Murphy, J.R., Skiver, J. and McBride, G. (1976). Isolation and partial characterization of a corynebacteriophage beta, tox operator constitutive-like mutant lysogen of *Corynebacterium diphtheriae.* J. Virol. ***18****, 235–244.*

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. (1992a). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051–1061.

Naglich, J.G., Rolf, J.M. and Eidels, L. (1992b). Expression of functional diphtheria toxin receptors on highly toxin-sensitive mouse cells that specifically bind radioiodinated toxin. *Proc. Natl. Acad. Sci. USA* **89**, 2170–2174.

Nakamura, K., Mitamura, T., Takahashi, T., Kobayashi, T. and Mekada, E. (2000). Importance of the major extracellular domain of CD9 and the epidermal growth factor (EGF)-like domain of heparin-binding EGF-like growth factor for up-regulation of binding and activity. *J. Biol. Chem*. **275**, 18284–18290.

Oh, K.J., Senzel, L., Collier, R.J. and Finkelstein, A. (1999a). Translocation of the catalytic domain of diphtheria toxin across planar phospholipid bilayers by its own T domain. *Proc. Natl. Acad. Sci. USA* **96**, 8467–8470.

Oh, K.J., Zhan, H., Cui, C., Altenbach, C., Hubbell, W. L. and Collier, R. J. (1999b). Conformation of the diphtheria toxin T domain in membranes: a site-directed spin-labeling study of the TH8 helix and TL5 loop. *Biochemistry* **38**, 10336–10343.

Oram, D.M., Avdalovic, A. and Holmes, R.K. (2004). Analysis of genes that encode DtxR-like transcriptional regulators in pathogenic and saprophytic corynebacterial species. *Infect. Immun*. **72**, 1885–1895.

Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxwell, F., Maxwell, I. H. and Brinster, R.L. (1987). Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* **50**, 435–443.

Papini, E., Sandona, D., Rappuoli, R. and Montecucco, C. (1988). On the membrane translocation of diphtheria toxin: at low pH the toxin induces ion channels on cells. *Embo J*. **7**, 3353–3359.

Papini, E., Santucci, A., Schiavo, G., Domenighini, M., Neri, P., Rappuoli, R. and Montecucco, C. (1991). Tyrosine 65 is photolabeled by 8-azidoadenine and 8-azidoadenosine at the NAD binding site of diphtheria toxin. *J. Biol. Chem*. **266**, 2494–2498.

Papini, E., Schiavo, G., Rappuoli, R. and Montecucco, C. (1990). Histidine-21 is involved in diphtheria toxin NAD+ binding. *Toxicon* **28**, 631–635.

Papini, E., Schiavo, G., Sandona, D., Rappuoli, R. and Montecucco, C. (1989). Histidine 21 is at the NAD+ binding site of diphtheria toxin. *J. Biol. Chem*. **264**, 12385–12388.

Pappenheimer, A.M., Jr. (1984). The diphtheria bacillus and its toxin: a model system. *J. Hyg. (Lond)* **93**, 397–404.

Pappenheimer, A.M.J. and Johnson, S. (1936). Studies on diphtheria toxin production. I. The effect of iron and copper. *Br. J. Exp. Pathol*. **17**, 335–341.

Passwell, J.H., Ashkenazi, S., Harlev, E., Miron, D., Ramon, R., Farzam, N., Lerner-Geva, L., Levi, Y., Chu, C., Shiloach, J. *et al*. (2003). Safety and immunogenicity of *Shigella sonnei*-CRM9 and *Shigella flexneri* type 2a-rEPAsucc conjugate vaccines in one- to four-year-old children. *Pediatr. Infect. Dis. J*. **22**, 701–706.

Pentz, E.S., Moyano, M.A., Thornhill, B.A., Sequeira Lopez, M.L. and Gomez, R.A. (2004). Ablation of renin-expressing juxtaglomerular cells results in a distinct kidney phenotype. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **286**, R474–483.

Phan, L.D., Perentesis, J.P. and Bodley, J.W. (1993). *Saccharomyces cerevisiae* elongation factor 2. Mutagenesis of the histidine precursor of diphthamide yields a functional protein that is resistant to diphtheria toxin. *J. Biol. Chem*. **268**, 8665–8668.

Placido Sousa, C. and Evans, D.G. (1957). The action of diphtheria toxin on tissue cultures and its neutralization by antitoxin. *Br. J. Exp. Pathol*. **38**, 644–649.

Pohl, E., Holmes, R.K. and Hol, W.G. (1999). Crystal structure of a cobalt-activated diphtheria toxin repressor-DNA complex reveals a metal-binding SH3-like domain. *J. Mol. Biol*. **292**, 653–667.

Qian, Y., Lee, J.H. and Holmes, R.K. (2002). Identification of a DtxR-regulated operon that is essential for siderophore-dependent iron uptake in *Corynebacterium diphtheriae*. *J. Bacteriol*. **184**, 4846–4856.

Quertenmont, P., Wattiez, R., Falmagne, P., Ruysschaert, J.M. and Cabiaux, V. (1996). Topology of diphtheria toxin in lipid vesicle membranes: a proteolysis study. *Mol. Microbiol*. **21**, 1283–1296.

Quertenmont, P., Wolff, C., Wattiez, R., Vander Borght, P., Falmagne, P., Ruysschaert, J.M. and Cabiaux, V. (1999). Structure and topology of diphtheria toxin R domain in lipid membranes. *Biochemistry* **38**, 660–666.

Ramon Jr., G. (1923). Sur le pouvoir floculant et sur les proprietis immunisantes d'une toxine diphtherique rendue anatoxique (anatoxine). Comptes rendues de la *Academie di Sciencis*, 1330–1338.

Rappuoli, R., Perugini, M. and Falsen, E. (1988). Molecular epidemiology of the 1984–1986 outbreak of diphtheria in Sweden. *N. Engl. J. Med*. **318**, 12–14.

Ratti, G., Rappuoli, R. and Giannini, G. (1983). The complete nucleotide sequence of the gene coding for diphtheria toxin in the corynephage omega (tox+) genome. *Nucleic Acids Res*. **11**, 6589–6595.

Ratts, R., Zeng, H., Berg, E.A., Blue, C., McComb, M.E., Costello, C. E., vanderSpek, J.C. and Murphy, J.R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J. Cell. Biol*. **160**, 1139–1150.

Ren, J., Kachel, K., Kim, H., Malenbaum, S.E., Collier, R.J. and London, E. (1999a). Interaction of diphtheria toxin T domain with molten globule-like proteins and its implications for translocation. *Science* **284**, 955–957.

Ren, J., Sharpe, J.C., Collier, R.J. and London, E. (1999b). Membrane translocation of charged residues at the tips of hydrophobic helices in the T domain of diphtheria toxin. *Biochemistry* **38**, 976–984.

Rodriguez, G.M. and Smith, I. (2003). Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. *Mol. Microbiol*. **47**, 1485–1494.

Rosconi, M.P., Zhao, G. and London, E. (2004). Analyzing topography of membrane-inserted diphtheria toxin T domain using BODIPY-streptavidin: at low pH, helices 8 and 9 form a transmembrane hairpin but helices 5–7 form stable nonclassical inserted segments on the cis side of the bilayer. *Biochemistry* **43**, 9127–9139.

Roux, E., Jr. and Yersin, A. (1888). Contribution a l'etude de la diphtherie. *Ann. Inst. Pasteur*. **2**, 620–629.

Rydell, N. and Sjoholm, I. (2004). Oral vaccination against diphtheria using polyacryl starch microparticles as adjuvant. *Vaccine* **22**, 1265–1274.

Ryu, F., Takahashi, T., Nakamura, K., Takahashi, Y., Kobayashi, T., Shida, S., Kameyama, T. and Mekada, E. (2000). Domain analysis of the tetraspanins: studies of CD9/CD63 chimeric molecules on subcellular localization and up-regulation activity for diphtheria toxin binding. *Cell. Struct. Funct*. **25**, 317–327.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A. and Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol*. **19**, 746–750.

Sandvig, K. and Olsnes, S. (1980). Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell. Biol*. **87**, 828–832.

Sandvig, K. and Olsnes, S. (1988). Diphtheria toxin-induced channels in Vero cells selective for monovalent cations. *J. Biol. Chem*. **263**, 12352–12359.

Sandvig, K., Sundan, A. and Olsnes, S. (1985). Effect of potassium depletion of cells on their sensitivity to diphtheria toxin and pseudomonas toxin. *J. Cell. Physiol*. **124**, 54–60.

Schmitt, M.P. and Holmes, R.K. (1991). Iron-dependent regulation of diphtheria toxin and siderophore expression by the cloned *Corynebacterium diphtheriae* repressor gene *dtxR* in *C. diphtheriae* C7 strains. *Infect. Immun*. **59**, 1899–1904.

Schmitt, M.P. and Holmes, R.K. (1993). Analysis of diphtheria toxin repressor-operator interactions and characterization of a mutant repressor with decreased binding activity for divalent metals. *Mol. Microbiol*. **9**, 173–181.

Schmitt, M.P. and Holmes, R.K. (1994). Cloning, sequence, and footprint analysis of two promoter/operators from *Corynebacterium diphtheriae* that are regulated by the diphtheria toxin repressor (DtxR) and iron. *J. Bacteriol*. **176**, 1141–1149.

Schmitt, M.P., Twiddy, E.M. and Holmes, R.K. (1992). Purification and characterization of the diphtheria toxin repressor. *Proc. Natl. Acad. Sci. USA* **89**, 7576–7580.

Schultz, D.C., Balasara, B.R., Testa, J.R. and Godwin, A.K. (1998). Cloning and localization of a human diphthamide biosynthesis-like protein-2 gene, DPH2L2. *Genomics* **52**, 186–191.

Sharpe, J.C. and London, E. (1999). Diphtheria toxin forms pores of different sizes depending on its concentration in membranes: probable relationship to oligomerization. *J. Membr. Biol*. **171**, 209–221.

Shen, W.H., Choe, S., Eisenberg, D. and Collier, R.J. (1994). Participation of lysine 516 and phenylalanine 530 of diphtheria toxin in receptor recognition. *J. Biol. Chem*. **269**, 29077–29084.

Shishido, Y., Sharma, K.D., Higashiyama, S., Klagsbrun, M. and Mekada, E. (1995). Heparin-like molecules on the cell surface potentiate binding of diphtheria toxin to the diphtheria toxin receptor/membrane-anchored heparin-binding epidermal growth factor-like growth factor. *J. Biol. Chem*. **270**, 29578–29585.

Simpson, J.C., Smith, D.C., Roberts, L.M. and Lord, J.M. (1998). Expression of mutant dynamin protects cells against diphtheria toxin but not against ricin. *Exp. Cell. Res*. **239**, 293–300.

Strauss, N. and Hendee, E.D. (1959). The effect of diphtheria toxin on the metabolism of HeLa cells. *J. Exp. Med*. **109**, 145–163.

Tao, X., Boyd, J. and Murphy, J.R. (1992). Specific binding of the diphtheria tox regulatory element DtxR to the tox operator requires divalent heavy metal ions and a 9-base-pair interrupted palindromic sequence. *Proc. Natl. Acad. Sci. USA* **89**, 5897–5901.

Tao, X. and Murphy, J.R. (1992). Binding of the metalloregulatory protein DtxR to the diphtheria tox operator requires a divalent heavy metal ion and protects the palindromic sequence from DNase I digestion. *J. Biol. Chem*. **267**, 21761–21764.

Tao, X. and Murphy, J.R. (1994). Determination of the minimal essential nucleotide sequence for diphtheria tox repressor binding by *in vitro* affinity selection. *Proc. Natl. Acad. Sci. USA* **91**, 9646–9650.

Tsugeki, R. and Fedoroff, N.V. (1999). Genetic ablation of root cap cells in *Arabidopsis. Proc. Natl. Acad. Sci. USA* ***96***, 12941–12946.

Tweten, R.K., Barbieri, J.T. and Collier, R.J. (1985). Diphtheria toxin. Effect of substituting aspartic acid for glutamic acid 148 on ADP-ribosyltransferase activity. *J. Biol. Chem*. **260**, 10392–10394.

Uchida, T., Gill, D.M. and Pappenheimer, A.M., Jr. (1971). Mutation in the structural gene for diphtheria toxin carried by temperate phage. *Nat. New. Biol*. **233**, 8–11.

Uchida, T., Kanei, C. and Yoneda, M. (1977). Mutations of corynephage beta that affect the yield of diphtheria toxin. *Virology* **77**, 876–879.

Uchida, T., Pappenheimer, A.M., Jr. and Greany, R. (1973). Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem*. **248**, 3838–3844.

Umata, T. and Mekada, E. (1998). Diphtheria toxin translocation across endosome membranes. A novel cell permeabilization assay reveals new diphtheria toxin fragments in endocytic vesicles. *J. Biol. Chem*. **273**, 8351–8359.

Uzbekova, S., Amoros, C., Cauty, C., Mambrini, M., Perrot, E., Hew, C. L., Chourrout, D. and Prunet, P. (2003). Analysis of cell-specificity and variegation of transgene expression driven by salmon prolactin promoter in stable lines of transgenic rainbow trout. *Transgenic Res*. **12**, 213–227.

Walker, P.L. and Dang, N.H. (2004). Denileukin diftitox as novel targeted therapy in non-Hodgkin's lymphoma. *Clin. J. Oncol. Nurs*. **8**, 169–174.

Welkos, S.L. and Holmes, R.K. (1981a). Regulation of toxinogenesis in *Corynebacterium diphtheriae*. I. Mutations in bacteriophage beta that alter the effects of iron on toxin production. *J. Virol*. **37**, 936–945.

Welkos, S.L. and Holmes, R.K. (1981b). Regulation of toxinogenesis in *Corynebacterium diphtheriae*. II. Genetic mapping of a tox regulatory mutation in bacteriophage beta. *J. Virol*. **37**, 946–954.

White, A., Ding, X., vanderSpek, J.C., Murphy, J.R. and Ringe, D. (1998). Structure of the metal-ion-activated diphtheria toxin repressor/tox operator complex. *Nature* **394**, 502–506.

Wiedlocha, A., Madshus, I.H., Mach, H., Middaugh, C.R. and Olsnes, S. (1992). Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. *Embo J*. **11**, 4835–4842.

Wilson, B.A., Blanke, S.R., Reich, K.A. and Collier, R.J. (1994). Active-site mutations of diphtheria toxin. Tryptophan 50 is a major determinant of NAD affinity. *J. Biol. Chem*. **269**, 23296–23301.

Wisedchaisri, G., Holmes, R.K. and Hol, W.G. (2004). Crystal structure of an IdeR-DNA complex reveals a conformational change in activated IdeR for base-specific interactions. *J. Mol. Biol*. **342**, 1155–1169.

Wolff, C., Wattiez, R., Ruysschaert, J.M. and Cabiaux, V. (2004). Characterization of diphtheria toxin's catalytic domain interaction with lipid membranes. *Biochim. Biophys. Acta*. **1661**, 166–177.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15**, 245–250.

Zhan, H., Choe, S., Huynh, P.D., Finkelstein, A., Eisenberg, D. and Collier, R. J. (1994). Dynamic transitions of the transmembrane domain of diphtheria toxin: disulfide trapping and fluorescence proximity studies. *Biochemistry* **33**, 11254–11263.

CHAPTER

# 14

# *Pseudomonas aeruginosa* toxins

*Anthony W. Maresso, Dara W. Frank, and Joseph T. Barbieri*

## INTRODUCTION

Bacterial pathogens subvert host responses through multiple and diverse mechanisms, while eukaryotic organisms utilize innate and acquired immunity to prevent bacterial pathogens from establishing infections. A successful pathogen must initially permeate a network of barrier-forming cells, down-regulate or eliminate an innate and directed adaptive immune response, replicate, and then damage the host. Each bacterial pathogen damages the host by unique molecular mechanisms. *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that has adapted to grow in diverse environments, from soil and water reservoirs to the mammalian lung. *P. aeruginosa* is an opportunistic pathogen that utilizes surface and secreted virulence factors, including a potent exotoxin and four type III secreted cytotoxins. The structural, molecular, and functional properties of these toxins will be discussed in this chapter.

*P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections (Van Delden and Iglewski, 1998). *P. aeruginosa* is pathogenic to individuals who suffer from immune system complications, such as neutropenics and burn victims, or genetic disorders such as cystic fibrosis (CF) (Adams *et al*., 1998). Intrinsic resistance to antibiotics makes *P. aeruginosa* difficult to eradicate (Finland, 1972; Mendelson *et al*., 1994; Kazama *et al*., 1998). Cell surface and secreted virulence factors include endotoxin, fimbriae, flagella, proteases, phospholipases, iron siderophores, and alginate (Barbieri, 2000). Cytotoxins include exotoxin A (ETA), a classic AB exotoxin, and four type III cytotoxins, ExoS, ExoT, ExoY, and ExoU (Figure, 1 & Table 14.1). Each cytotoxin contributes uniquely to the pathogenic potential of *P. aeruginosa*.

### Exotoxin A

*P. aeruginosa* produces three ADP-ribosyltransferases: ETA, ExoS, and ExoT. Both ETA and ExoS contribute to pathogenesis, since strains of *P. aeruginosa* that are deleted for the genes encoding these proteins are less toxic than parental strains (Nicas *et al*., 1985; Nicas and Iglewski, 1985). ExoT has been linked to an anti-internalization activity of *P. aeruginosa* (Garrity-Ryan *et al*., 2000).

#### *Identification and AB structure-function properties of ETA*

Shortly after the determination that diphtheria toxin ADP-ribosylated elongation factor-2 (EF-2), ETA was isolated from the culture supernatant of *P. aeruginosa* and also shown to ADP-ribosylate EF-2 (Iglewski *et al*., 1977). ETA was initially cloned from the chromosome of *P. aeruginosa* strain PA103 and determined to encode a 638 amino acid protein that included a 30 amino acid N-terminal signal sequence (Gray *et al*., 1984). Unlike diphtheria toxin, which encoded an N-terminal catalytic domain, the catalytic domain of ETA was located in the C terminus (Figure 14.2) (Hwang *et al*., 1987). Unexpectedly, diphtheria toxin and ETA shared little primary amino acid homology. Subsequent studies showed that this is a general property of bacterial ADP-ribosylating exotoxins (bAREs). Despite having conserved structural active sites, these toxins share limited overall conservation of structure within the catalytic domain and possess a limited number of

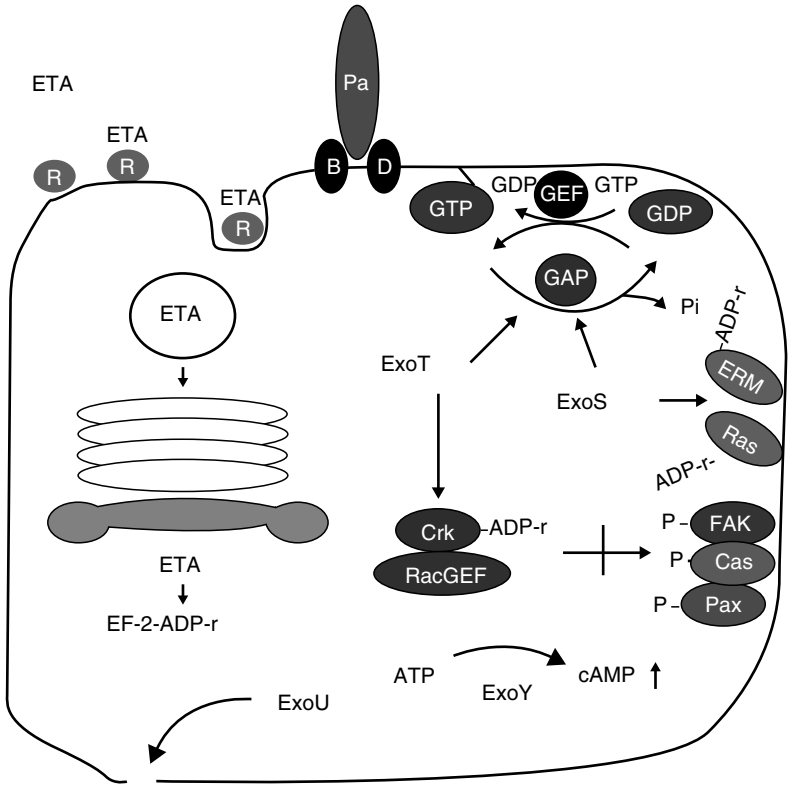



**FIGURE 14.1** *P. aeruginosa* translocates five toxins into eukaryotic cells. ETA is an AB exotoxin that is internalized via receptor-mediated endocytosis and transported to the ER. Upon release into the cytosol, ETA ADP-ribosylates EF-2. ExoS, ExoT, ExoY, and ExoU are type III delivered toxins that possess unique enzymatic activities. ExoS is poly-ADP-ribosylation specific and ADP-ribosylates numerous host proteins, including small molecular weight GTPases and the ERM proteins. ExoT ADP-ribosylates CrkI/II. The RhoGAP domain of ExoS and ExoT inactivate Rho, Rac, and Cdc42. The adenylyl cyclase activity of ExoY leads to supra-physiological levels of cAMP. ExoU is the most potent type III cytotoxin of *P. aeruginosa* that acts as a phospholipase.

**TABLE 14.1** Properties of *P. aeruginosa* toxins

| Toxin | Mw, pI, AA[a] | Enzymatic activity | Substrates[b] | Co-factor | Delivery | Other domains[c] |
|---|---|---|---|---|---|---|
| ETA | 68, 5.4, 638 | ADP-r | EF2 | No | receptor-mediated endocytosis | Leader sequence (1–30)<br>Receptor binding (1–252)<br>Translocation (253–364) |
| ExoS | 49, 5.5, 453 | ADP-r, GAP | ADP-r (GTPase, ERM), GAP (GTPase) | Yes (Fas) | type III | Secretion (1–15)<br>Chaperone (15–50)<br>MLD (51–77) |
| ExoT | 52, 5.5, 456 | ADP-r, GAP | ADP-r (Crk I/II), GAP (GTPase) | Yes (Fas) | type III | Secretion (1–15)<br>Chaperone (15–50)<br>MLD (51–77) |
| ExoY | 42, 5.4, 378 | Adenylate cyclase | NA | Yes (unknown) | type III | Secretion (1–15)<br>Chaperone (15–50) |
| ExoU | 74, 5.7, 687 | Phospholipase | Neutral/ Phospholipids | Yes (unknown) | type III | Secretion (1–15)<br>Chaperone (15–50) |

[a]Mw is in kDa, AA refers to the number of amino acids.
[b]ExoS ADP-ribosyltransferase can also modify vimentin(Coburn *et al.*, 1989a), apolipoprotein 1A, IgG (Knight and Barbieri, 1997), and the artificial substrate soybean trypsin inhibitor (SBTI) (Liu *et al.*, 1996).
[c]The secretion signal of ExoT, ExoY, ExoU, and the chaperone binding domain of ExoS, ExoT, and ExoY, and ExoU are inferred by analogy to *Yersinia* cytotoxins. The MLD of ExoT is inferred by analogy to ExoS.

conserved residues that are involved in catalysis. bAREs have AB structure-function properties; the A domain comprises the ADP-ribosyltransferase activity, while the B domain includes the receptor binding domain and the translocation domain, which delivers the A domain into eukaryotic cells (Figures 14.1 and 14.2). The three domains of ETA are discrete structures with an N-terminal receptor binding domain, an internal translocation domain, and C-terminal ADP-ribosyltransferase domain (Figure 14.2) (Allured *et al*., 1986).

### *Entry and intoxication of eukaryotic cells by ETA*

ETA expression is regulated by several factors, including iron (Carty *et al*., 2003). ETA is secreted from *P. aeruginosa* into the surrounding environment by the type II secretion pathway, which requires cleavage of an N-terminal signal sequence for export (McVay and Hamood, 1995). ETA binds the low-density lipoprotein receptor-related (LRP) protein on the surface of sensitive eukaryotic cells (Kounnas *et al*., 1992) and is internalized via receptor-mediated endocytosis. Like diphtheria toxin, ETA is activated by proteolytic processing and disulfide bond reduction. Furin, or a furin-like enzyme, cleaves ETA on the cell surface or after entry into the endosome on the C-terminal side of RQPR (amino acids 276–279 of ETA) (Chiron *et al*., 1994; Inocencio *et al*., 1994) and after disulfide bond reduction to generate a 37 kDa catalytic A domain (Ogata *et al*., 1990; Ogata *et al*., 1992). ETA possesses a C-terminal signal sequence, REDLK, which is functionally analogous to the eukaryotic KDEL sequence (Chaudhary *et al*., 1990). Upon cleavage of the C-terminal lysine, ETA retrograde traffics from the early endosome to the endoplasmic reticulum (ER). Similar to cholera toxin (Feng *et al*., 2004), the A domain of ETA is secreted from the lumen of the ER into the cytosol by the Sec63, a complex that eliminates improperly folded proteins from the lumen of the ER (Willer *et al*., 2003). The A domain ADP-ribosylates EF-2, blocking mRNA translocation within the ribosome complex, inhibiting eukaryotic cell protein synthesis (Lee and Iglewski, 1984).

Although the A domains of diphtheria toxin and ETA possess limited primary amino acid homology, the toxins share a common reaction mechanism that utilizes a conserved catalytic glutamic acid. Like Glu-149 of diphtheria toxin (Carroll *et al*., 1985), Glu-553 of ETA is necessary for ADP-ribosyltransferase activity (Carroll and Collier, 1987; Douglas and Collier, 1990).

## TYPE III SECRETION IN *P. AERUGINOSA*

*P. aeruginosa* delivers four cytotoxins directly into the host cell by the type III secretion system (Frank, 1997). The secretion and translocation of the *Pseudomonas* type III cytotoxins is analogous to the type III secretion system in *Yersinia* (Cornelis, 2002b). The genes encoding

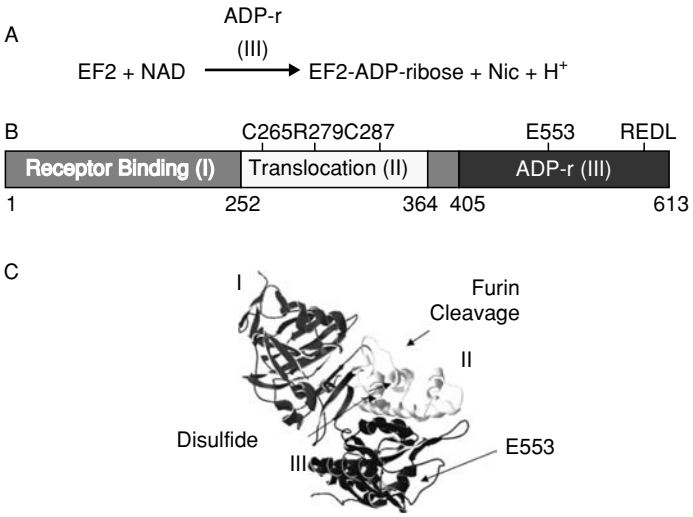



**FIGURE 14.2** Enzymatic activity, domain organization, and structure of ETA. A-B ETA is an AB exotoxin with three functional domains: an N-terminal receptor binding domain (1–252), an internal translocation domain (252–346), and a C-terminal ADP-ribosyltransferase domain (405–613). Glu-553 is a catalytic residue required for ADP-r activity. Cys-265 and Cys-287 form a disulfide bond that is reduced following proteolysis at Arg-279. **C** The crystal structure of ETA showing the three-dimensional arrangement of the domains (PDB, 1IKQ) (Wedekind *et al*., 2001).

the type III secretion system are clustered on the *P. aeruginosa* chromosome and designated *psc*, *pcr*, *exs*, and *pop*. *Psc* and *pcr* encode components of the type III secretion apparatus and *exs* are transcriptional regulators of the system (Frank, 1997). The type III apparatus is composed of 20 or more proteins with a diverse range of functions, which includes an ATPase (PscN) that provides energy for translocation and chaperones that maintain translocation competent conformation of the effectors in the cytoplasm of the bacterium. In addition to the type III apparatus, three proteins, PopB, PopD, and PcrV, provide passage of the type III cytotoxins across the eukaryotic membrane. Conservation of function between PopB, PopD, and PcrV and their *Yersinia* homologies underscore the conservation function of the type III apparatus of the two pathogens (Frithz-Lindsten *et al*., 1998; Broms *et al*., 2003).

## ExoS and ExoT

### *Identification of exoenzyme S*

The earliest studies of ExoS were performed on a protein complex that was termed e*xoenzyme S*. Exoenzyme S was initially identified as a second ADP-ribosyltransferase of *P. aeruginosa*, based upon the ability to ADP-ribosylate proteins that were distinct from EF-2, the preferred target for ADP-ribosylation by ETA (Iglewski *et al*., 1978). Exoenzyme S ADP-ribosylated many cellular proteins and was considered a broad specificity ADP-ribosyltransferase (Coburn *et al*., 1989b). exoenzyme S was purified from the spent culture media of *P. aeruginosa* strain 388 as an aggregate composed of two proteins with molecular masses of 53 kDa and 49 kDa. The 49-kDa form of ExoS possessed ADP-ribosyltransferase activity, while the 53-kDa form of exoenzyme S lacked detectable ADP-ribosyltransferase activity (Nicas and Iglewski, 1984). The genes encoding the 49-kDa and 53-kDa proteins were subsequently cloned and designated ExoS for the 49-kDa protein and ExoT for the 53-kDa protein (Kulich *et al*., 1994). Subsequent studies determined that while ExoS was a polysubstrate-specific ADP-ribosyltransferase, ExoT ADP-ribosylated a limited subset of mammalian proteins, including the Crk proteins (Sun and Barbieri, 2003). FASTA alignment predicts that the ExoS ADP-ribosyltransferase domain is more similar to eukaryotic ADP-ribosyltransferases than bacterial ADP-ribosyltransferases (Ganesan *et al*., 1999a), which is consistent with the broad substrate specificity observed for some eukaryotic ADP-ribosyltransferases (Okazaki and Moss, 1996).

ExoS (453 amino acids) and ExoT (457 amino acids) are bifunctional cytotoxins that possess linear functional domains. The N terminus encodes the type III recognition sequence and the chaperone binding domain; residues 51–77 comprise a membrane localization domain (MLD) that targets type III delivered ExoS and ExoT within the mammalian cell; residues ~96–219 comprise a Rho GAP domain, and the C-terminal ~220 residues comprise the ADP-ribosyltransferase domain.

### *Membrane localization domain (MLD)*

During early studies, ExoS purified from the culture supernatants of *P. aeruginosa* as an aggregate of >300 kDa (Kulich *et al*., 1993). This was consistent with a domain within ExoS that mediated self-association. Further studies identified a hydrophobic stretch of amino acids between residues 51–72 (subsequently termed the *membrane localization domain*, MLD) of ExoS that was responsible for the aggregative properties and for the localization of ExoS to a membrane fraction after delivery into mammalian cells. Corroborative studies showed that MLD targeted ExoS to a Golgi-ER-like region inside mammalian cells (Pederson *et al*., 2002). Deletion of the MLD resulted in the loss of ExoS aggregation and the homogenous purification of the toxin in *E. coli* (Maresso and Barbieri, 2002), suggesting that the MLD was also responsible for the self-association. Deletion of the MLD also facilitated the study of ExoS-specific conformers, a charge heterogeneity of ExoS observed by 2D-electrophoresis thought to be related to the ability to transverse lipid bilayers upon type III translocation (Maresso *et al*., 2003). The MLD targeted ExoS to Ras, ExoS that lacked the MLD modified Ras inefficiently *in vivo,* but not *in vitro* (Riese and Barbieri, 2002). A leucine-rich motif within the MLD was responsible for targeting ExoS to the Golgi-ER region of mammalian cells (Zhang and JTB, submitted).

### *RhoGAP Domain of ExoS and ExoT*

The RhoGAP domains of ExoS and ExoT are functionally identical. An early indication that ExoS possessed a second catalytic activity was predicted from the determination that mutated ExoS that lacked ADP-ribosyltransferase activity retained partial capacity to stimulate the reorganization of the actin cytoskeleton (Frithz-Lindsten *et al*., 1997). Subsequent studies showed that a peptide comprising the N-terminal 234 amino acids of ExoS stimulated the reorganization of the actin cytoskeleton (Pederson and Barbieri, 1998). This morphological change was reversed by CNF1, a known activator of Rho, implicating the inactivation of the Rho pathway (Pederson *et al*., 1999). *In vitro* studies identified ExoS(1-234) as a Rho GTPase Activating Protein or (GAP) that facilitated the inactivation of Rho, Rac, and Cdc42 by hydrolyzing the γ–phosphate from GTP-bound Rho (Figure 14.3). Similar to the

mammalian RhoGAPs, ExoS RhoGAP utilized a catalytic arginine (Arg-146) to stimulate GAP activity (Figure 14.3) (Goehring *et al*., 1999). The GAP domain of ExoS has little structural homology to the mammalian p50RhoGAP, but utilizes a similar mechanism to stimulate GTPase activity. The p50RhoGAP inserts a catalytic arginine into the active site of the Rho GTPase to indirectly stabilize a catalytic $H_2O$ in the transition state of the GAP reaction (Wurtele *et al*., 2001), while ExoS RhoGAP inserts the primary chain carboxyl group of Gly141 to indirectly stabilize the catalytic $H_2O$. In cultured cells, Rho, Rac, and Cdc42 were inactivated by type III delivered ExoS Rho GAP (Krall *et al*., 2002), promoting deregulation of the actin cytoskeleton, actin stress fiber formation, and cell rounding. YopE, the *Yersinia* homologue to the RhoGAP domain of ExoS, prevented phagocytosis of *Yersinia* (Black and Bliska, 2000), supporting a role for the inactivation of the Rho GTPases in the down-regulation of phagocytosis.

ExoT was a GAP for Cdc42, Rac, and Rho *in vitro* and elicited cell rounding *in vivo*, presumably through the same mechanism as ExoS (Krall *et al*., 2000; Pederson *et al*., 2002). Arg-149 (Figure 14.3B) is required for the Rho GAP activity of ExoT. The Rho GAP activity of ExoT prevents phagocytosis of *P. aeruginosa* (Garrity-Ryan *et al*., 2000).

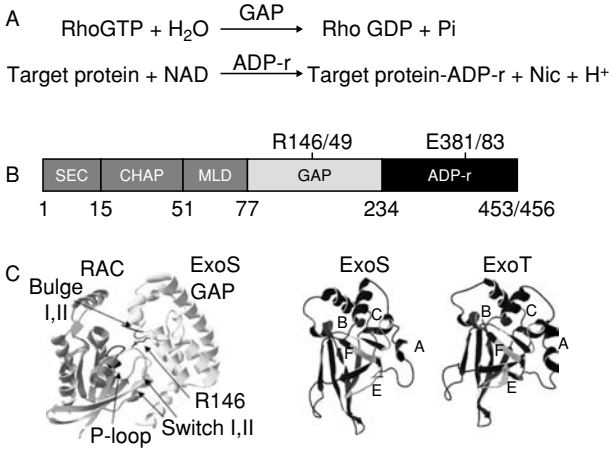



**FIGURE 14.3** Enzymatic activity, domain organization, and structures of ExoS and ExoT. (A-B) ExoS/T Rho GAP activity (96-234) act to stimulate the hydrolysis of the terminal phosphate from GTP bound Rho GTPases to make RhoGDP, thus inactivating the Rho pathway. ExoS/T ADP-ribosyltransferase activity (ExoS/ExoT) (235-453/456) covalently attaches and ADP-ribose moiety from NAD onto target substrates. (C) Left panel: The co-crystal of the complex between the Rho GAP domain of ExoS (right) and Rac (left), showing the catalytic arginine (146) inserting into the active site of Rac (PDB, 1HE1) (Wurtele *et al*., 2001). Bulge one and two regions, important in coordinating Arg-146, are shown. The P-loop, important for nucleotide binding, and the switch I and II regions, important in the intrinsic GTPase reaction of Rac, are indicated. Right panel: Modeled three-dimensional structures of ExoS and ExoT (Sun, *et al*., 2004).

### *ADP-ribosyltransferase domain of ExoS*

The ADP-ribosyltransferase domain lies within the C-terminal 220 amino acids of ExoS (Knight *et al*., 1995). ExoS is a biglutamic acid transferase; Glu-379 contributes to the transfer of ADP-ribose to target proteins, while Glu-381 is required for catalysis (Liu *et al*., 1996; Radke *et al*., 1999). Mutation of Glu-381 to aspartic acid-reduced catalysis ~2000-fold (Liu *et al*., 1996).

The first substrate of ExoS was vimentin, an actin cytoskeleton-associated protein (Coburn *et al*., 1989a). Subsequently, ExoS was shown to ADP-ribosylate numerous mammalian proteins, including several small-molecular-weight monomeric GTPases and itself (Coburn and Gill, 1991; Riese *et al*., 2002). Ras is the most extensively characterized substrate of ExoS. Ras regulates several cellular signal transduction pathways that are involved in differentiation, growth, and survival (Wiesmuller and Wittinghofer, 1994). ExoS ADP-ribosylates Ras at two arginines. Arg-41 is the preferred site and Arg-123 is the secondary site for ADP-ribosylation. Arg-41 and Arg-123 lie on distinct surfaces of Ras, indicating that ExoS can bind Ras in multiple conformations (Ganesan *et al*., 1998). ADP-ribosylation at Arg-41 blocks the association of Ras with its cognate *G*uanine *E*xchange *F*actor (GEF), preventing the activation of Ras (Ganesan *et al*., 1999b). Type III delivered ExoS ADP-ribosylates Ras in cultured cells (McGuffie *et al*., 1998; Henriksson *et al*., 2002; Riese and Barbieri, 2002), suggesting a functional role for Ras ADP-ribosylation *in vivo*.

ExoS also ADP-ribosylates Rap (Riese *et al*., 2001), RalA (Fraylick *et al*., 2002), Rac, Rab, and Cdc42 (Henriksson *et al*., 2002). Like Ras, ADP-ribosylation uncouples the interaction Rap with its cognate GEF, C3G (Riese *et al*., 2001), suggesting that ExoS uses a common inhibition mechanism to modulate host signal transduction pathways. Recently, ExoS has been shown to ADP-ribosylate the ezrin/radixin/moesin (ERM) proteins with high affinity both *in vitro* and during infection (Maresso *et al*., 2004). ERMs are regulators of Rho activation and are bridges between the actin cytoskeleton and receptor complexes. Inhibition of ERM function could lead to a loss of actin-rich structures, such as microvilli or down-regulation of the Rho signaling pathway (Mangeat *et al*., 1999).

### *ADP-ribosyltransferase domain of ExoT*

ExoT was originally proposed to be a precursor of ExoS. ExoT and ExoS were subsequently shown to be encoded by separate genes. ExoT ADP-ribosylated several substrates of ExoS at ~0.2% of the rate of ExoS

and appeared to encode a defective enzyme (Liu *et al*., 1997), despite sharing 76% primary amino acid homology with ExoS. The limited ADP-ribosyltransferase activity of ExoT required two glutamic acids, Glu-383 and Glu-385 (Figure 14.3B). Recent studies showed that ExoT elicited morphological changes to cultured cells, distinct from ExoS (Sun and Barbieri, 2003) and independent of Ras signal transduction (Sundin *et al*., 2001). ExoT was then implicated in the inhibition of lung epithelial wound repair (Geiser *et al*., 2001), and ExoT mutated for GAP activity retained anti-internalization activity (Garrity-Ryan *et al*., 2000), suggesting the ADP-ribosyltransferase domain had an unidentified function. These observations stimulated a more detailed characterization of the ADP-ribosyltransferase activity, which led to the determination that ExoT efficiently ADP-ribosylated the Crk proteins (Sun and Barbieri, 2003). Crk proteins contain SH2 and SH3 domains and are adaptors at focal adhesion sites, promoting cytoskeleton dynamics through the integrin pathway. Upon stimulation of the integrin receptors, Src kinase phosphorylates paxillin, focal adhesion kinase, and p130Cas within the focal adhesion complex. Paxillin-P and p130Cas-P bind the Crk SH2 domain, while the Crk SH3 domain binds to downstream exchange factors, the Rac GEF, DOCK180, and the Rap GEF, C3G (Tanaka *et al*., 1994). ADP-ribosylation of Crk I and Crk II may inhibit upstream interactions with Paxillin-P and p130Cas-P or downstream interactions with C3G or DOCK180, which may be a mechanism of *P. aeruginosa* ExoT-mediated anti-phagocytic action.

### *Substrate recognition by ExoS and ExoT ADP-r domains*

One of the intriguing questions in *P. aeruginosa* toxin biology is why both ExoS and ExoT ADP-ribosylate such diverse sets of host proteins. Diphtheria toxin, *E. coli* heat-labile enterotoxin, ETA, Pertussis toxin, and C3 exoenzyme have overall structural homology within the NAD binding cleft (Han *et al*., 2001). The C3 structure, which ADP-ribosylates Rho GTPases (Chardin *et al*., 1989; Mohr *et al*., 1992), has two adjacent turns within the NAD binding cleft that are predicted to be important for substrate recognition (Aktories *et al*., 1995). The first turn coordinates Phe-209 to interact with a hydrophobic stretch in the active site of Rho, while the second turn orients Gln-212 to hydrogen bond with Asn-41 of Rho for the nucleophilic attack on the NAD-ribose. This reaction mechanism was supported by mutations in Tyr-175 (Phe-209 of C2) of C3stau, which reduced ADP-ribosyltransferase activity (Wilde *et al*., 2002). The structures of ADP-ribosylating toxins Iota (Tsuge *et al*., 2003) and Vip2 (Han *et al*., 1999) define an ADP-ribosylating toxin-turn turn (ARTT) motif for substrate recognition.

Molecular modeling of the ADP-ribosyltransferase domains of ExoS and ExoT defined several regions that contribute to substrate recognition. Three loops surrounding the site of NAD binding defined ExoS substrate specificity, while an α-helix adjacent to the site of NAD binding defined the ExoT substrate specificity (Figure 14.3). Thus, although the overall shape of the active sites between ExoS and ExoT were similar (Figure 14.3C), the electrostatic potential appears to define substrate recognition with the polysubstrate specific ExoS being a mixture of acidic, basic, and neutral residues, while the Crk specific ExoT contains only an acidic surface (Sun *et al*., 2004).

## ExoY

### *ExoY, an adenylyl cyclase secreted directly into eukaryotic cells*

Comparison of extracellular protein profiles from parental and type III secretory mutants combined with the availability of genomic sequence facilitated the identification of a 42-kDa protein (378 amino acids), termed ExoY (Yahr *et al*., 1997; Yahr *et al*., 1998). The coordinate regulation of ExoY synthesis with other type III proteins, its extracellular localization, and the absence of a signal peptide sequence indicated ExoY as type III secreted protein (Yahr *et al*., 1998). ExoY contains two conserved regions (Regions I and II) that align with the adenylyl cyclases from *Bordetella pertussis* (CyaA) and *Bacillus anthracis* (edema factor, EF) (Escuyer *et al*., 1988; Mock *et al*., 1988). Region I, an ATP/GTP binding A motif, is postulated to be a contact point for the α-phosphate of the bound nucleotide (Glaser *et al*., 1991). Region II is common to various nucleotide-binding proteins and mediates interaction between the β and γ phosphates (Glaser *et al*., 1991). CyaA and EF both possess an additional region of homology (Region III) and a calmodulin binding site, which is absent in ExoY (Yahr *et al*., 1998). These data indicate that ExoY is a bacterial adenylyl cyclase delivered directly into eukaryotic cells by the type III secretion system of *P. aeruginosa*.

### *Enzymatic activity of ExoY*

Adenylyl cyclase activity of ExoY was demonstrated *in vitro* using a recombinant protein and HPLC separation of reaction products. The formation of cAMP from ATP was sensitive to phosphodiesterase

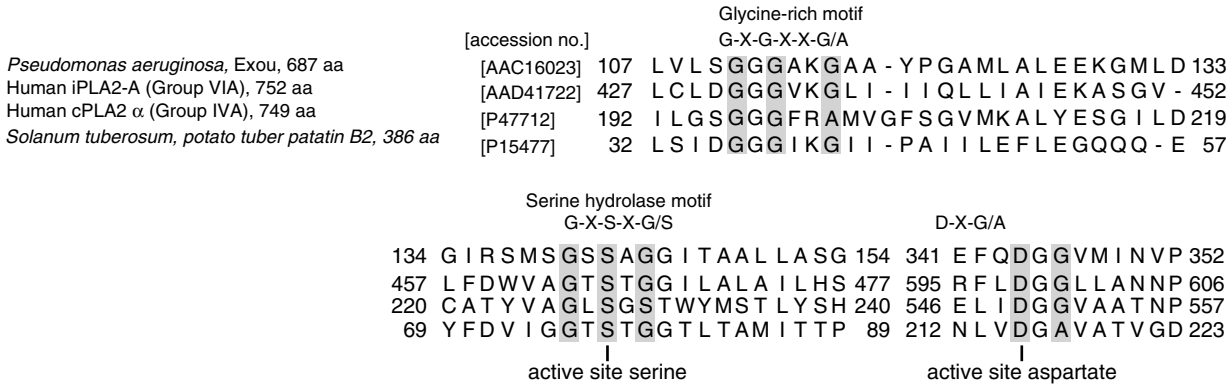



**FIGURE 14.4** Enzymatic activity and domain organization of ExoU. The alignment of ExoU (amino acids 107–133, 134–154, and 341–352) with the patatin-like phospholipase $A_2$ domains of human $iPLA_2$ (Group VIA), 752 aa; human $cPLA_2$ (Group IVA), 749 aa; and potato tuber patatin B2, 396 aa. Conserved amino acid residues among listed proteins are shaded and the catalytic amino acids are labeled.

inhibitors. Mutagenesis of conserved amino acid residues predicted to be involved in ATP binding (K81M, K88I, Region I and D212N, D214N, Region II) abolished *in vitro* adenylyl cyclase activity of ExoY. Substitution of GTP for ATP did not result in the formation of cGMP. Unlike CyaA and EF, calmodulin did not stimulate ExoY adenylyl cyclase activity, which is consistent with the absence of a calmodulin-binding domain in ExoY. The basal adenylyl cyclase activity of ExoY, however, was stimulated approximately 500-fold by a cytosolic extract of an epithelial cell line, Chinese hamster ovary (CHO) cells. The eukaryotic cofactor appears to be heat labile, but has not yet been identified.

### *Biological consequences of ExoY intoxication*

*In vivo*, ExoY is delivered into cultured cells by the type III secretion system (Yahr *et al*., 1998; Sawa *et al*., 1999). Mutations in proteins involved in type III translocation or secretion inhibit ExoY intoxication. Type III delivered ExoY intoxicates CHO cells by increasing intracellular cAMP, which correlates with a rounded cell morphology. Treatment of CHO cells with pertussis toxin or heat-labile enterotoxin also induces intracellular cAMP synthesis, but results in a different morphological change characterized as clustering. The differential changes in the cytoskeletal architecture may be related to the localization of ExoY relative to other toxins or to effects on endogenous mammalian adenylyl cyclases. In pulmonary microvascular endothelial cells (PMVEC), synthesis of cAMP increases tight junction complexes at cell-to-cell borders and prevents intercellular gap formation that occurs during inflammatory processes. This phenomenon is due to membrane-associated adenylyl cyclases that increase cAMP concentrations in plasma membrane microdomains. The pool of cAMP synthesized by ExoY that is injected during infection appears distinct from the endogenous pool of cAMP associated with the barrier function of endothelial cells (Sayner *et al*., 2004). Evidence to support this model includes the predominant cytoplasmic localization of ExoY during infection of PMVEC and the insensitivity of the ExoY-mediated cAMP pool to *in vivo* phosphodiesterase activity. In perfused lungs, ExoY expression correlates to increased endothelial permeability (Sayner *et al*., 2004). These data indicate that unlike the membrane-associated pools of cAMP that protect barrier function, injection and expression of cytoplasmically localized ExoY disrupts cytoskeletal components to result in intercellular gaps. Further, the insensitivity of cytoplasmic cAMP to phosphodiesterase regulation augments the longevity of the biologic response. Thus, expression of ExoY *in vivo* may increase fluid accumulation in tissues and worsen inflammation.

## ExoU

### *Differential phenotypes suggest that another cytotoxin is associated with the type III secretory system, the discovery of ExoU*

When administered to animals or in tissue culture infections, *P. aeruginosa* demonstrates observable variations in cytotoxicity and virulence. This property was used to identify ExoU, a potent phospholipase that is injected into target cells by the type III system. The original observation leading to the discovery of ExoU

showed that some strains of *P. aeruginosa* caused an acute cytotoxic response and cleared cultured cells within a few hours of infection (Fleiszig *et al*., 1997). The toxic activity was linked to the type III system due to its dependence on the transcriptional activator, ExsA, but was unrelated to ExoS, ExoY, or ExoT expression (Finck-Barbancon *et al*., 1997; Fleiszig *et al*., 1997; Yahr *et al*., 1998). All the strains that induced the acute cytotoxic response secreted a 72–74 kDa protein after growth in calcium-limited medium, a condition that induces type III secretion in *P. aeruginosa* (Finck-Barbancon *et al*., 1997). Transposon mutagenesis identified strains that possessed a specific chromosomal insertion within *exoU* (Finck-Barbancon *et al*., 1997; Kang *et al*., 1997; Hauser *et al*., 1998). Transposon mutants and strains specifically engineered to delete *exoU* no longer caused acute cytotoxicity and were complemented for this phenotype when *exoU* was provided in *trans*.

### *The mechanism of action of ExoU*

Determining the mechanism of action of ExoU was difficult. Transfection experiments suggested that minimal expression of ExoU caused cell death (Finck-Barbancon and Frank, 2001). When different subclones were used in co-transfection studies, several regions of ExoU appeared to be required but not sufficient for cytotoxicity. Controlled expression of ExoU in *Saccharomyces cerevisiae*, however, provided a genetically tractable model system with commercially available immunological reagents to accurately determine the biological events preceding cell death (Sato *et al*., 2003). These experiments revealed that ExoU was responsible for a vacuolar fragmentation phenotype. In addition, ExoU appeared to be affecting other organelles, as immunofluorescent signal intensity increased for markers located in mitochondria, late Golgi, and endosomes (Sato *et al*., 2003). Various inhibitors were tested to determine if ExoU cytotoxicity could be suppressed (Phillips *et al*., 2003; Sato *et al*., 2003). These studies indicated that irreversible inhibitors of calcium-independent ($iPLA_2$) and cytosolic ($cPLA_2$) phopholipase $A_2$ prevented cytotoxicity and allowed immunologic detection of full-length ExoU. The range of biologic effects and the specificity of the inhibitors indicated that ExoU likely possessed phospholipase activity.

ExoU aligns with patatin, a soluble storage protein of potato tubers, human $cPLA_2$, $iPLA_2$, as well as a number of bacterial encoded enzymes (Sato and Frank, 2004). The alignment of ExoU with patatin, $cPLA_2$, and $iPLA_2$ identified serine 142 and aspartate 344 as potential catalytic amino acids. Site-specific mutagenesis of S142 and D344 to alanine suppresses toxicity, supporting

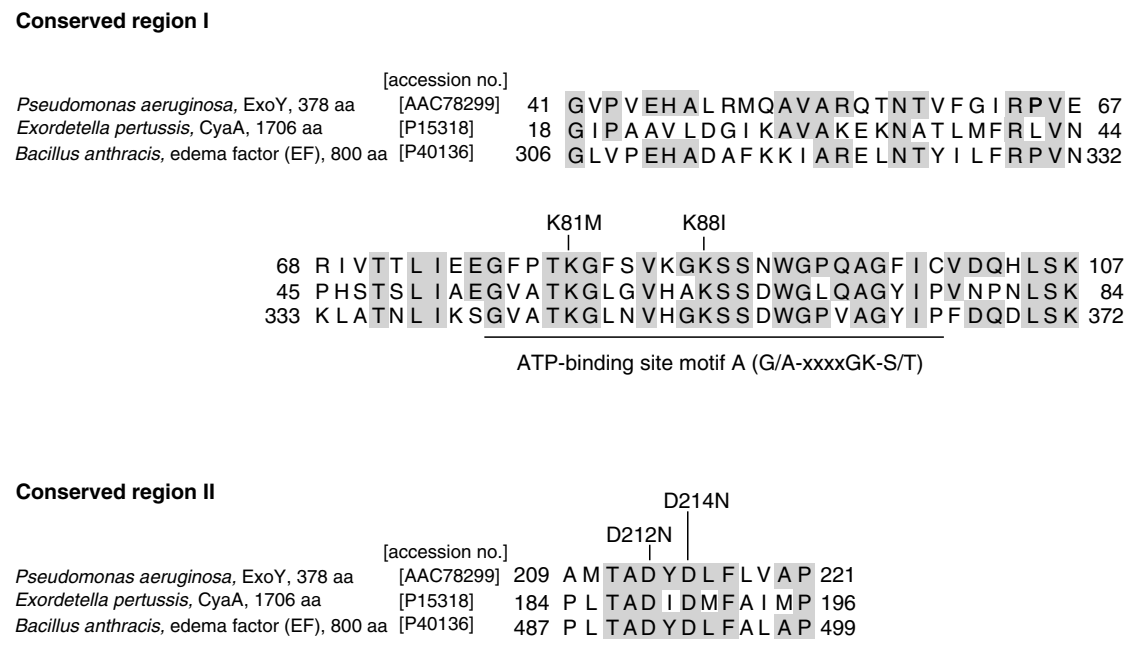



**FIGURE 14.5** Enzymatic activity and domain organization of ExoY. PILEUP alignment of the conserved regions I and II within ExoY, the adenylate cylase toxin from *Bordetella pertussis* (CyaA), and the edema factor from *Bacillus anthracis* (EF) adenylate cyclases are shown. Residues within CyaA and EF that are homologous to ExoY are shaded. Position of the ATP-binding site motif is shown.

the prediction that ExoU belongs to the family of enzymes utilizing a catalytic dyad (Phillips *et al*., 2003; Sato *et al*., 2003). These data suggest that the amino-terminal half of ExoU contains the catalytic domain. The catalytic domain of $cPLA_2$ is composed of the serine-aspartate dyad and a flexible lid that requires interfacial activation to access phospholipid substrates (Nalefski *et al*., 1994). ExoU, however, does not appear to possess a calcium-dependent lipid-binding domain. It is also unclear whether ExoU contains a flexible lid or requires interfacial activation.

A host protein also appears to activate phospholipase activity of ExoU. While *in vivo* expression of ExoU is detectable in yeast and mammalian cells by measuring the changes in phospholipid profiles upon induction (Phillips *et al*., 2003; Sato *et al*., 2003; Sato and Frank, 2004; Sato *et al*., 2005), the activity of purified recombinant protein was detectable only in the presence of crude yeast or mammalian cell extracts (Sato *et al*., 2003; Sato and Frank, 2004). These data indicate that similar to ExoS, ExoT, and ExoY, a cofactor is required for ExoU phospholipase activity. The cofactor appears to be limited to eukaryotic extracts, as prokaryotic lysates do not activate ExoU. ExoU will hydrolyze liposomes that simulate bacterial membranes, indicating that the apparent specificity of ExoU for eukaryotic membranes is related to the presence of the cofactor. Biochemically, the cofactor is susceptible to heat denaturation and to proteases, implicating that part of this activity is due to a protein or a protein complex (Sato *et al*., 2005).

### Mammalian proteins are cofactors for the *Pseudomonas* type III cytotoxins

Each of the four type III cytotoxins of *P. aeruginosa* utilize a mammalian protein to stimulate their activity. ExoS and ExoT ADP-ribosyltransferase activity is dependent on a eukaryotic cofactor termed factor activating ExoS (FAS) (Coburn *et al*., 1991). FAS is a member of the 14-3-3 family of ubiquitous proteins (Fu *et al*., 1993), which have diverse functions ranging from being scaffolds for signal transduction cascades to apoptosis. The exact mechanism by which ExoS is activated by FAS is unknown, but appears to be allosteric in action (Knight and Barbieri, 1999). The interaction site of FAS and ExoS is through an amphiphatic region on FAS (Masters *et al*., 1999) and a DALDA sequence at the C terminus of ExoS (Hallberg, 2002), which may mimic a negative charge normally present on eukaryotic proteins that bind FAS. ExoU (Sato *et al*., 2003) and ExoY (Yahr *et al*., 1998) activities are also stimulated by the addition of a mammalian cell lysate, implying that like ExoS and ExoT, they utilize a mammalian protein to activate their intrinsic catalytic activity. The utilization of eukaryotic cofactors by the *Pseudomonas* type III cytotoxins implies the importance of the posttranslational regulation of these enzyme activities. This may be related to the fact that these cytotoxins are synthesized and maintained in the cytosol of the *Pseudomonas*, presumably in full-length, catalytically active forms prior to secretion through the type III secretion apparatus.

## DISCUSSION

#### *Role of* Pseudomonas *cytotoxins in pathogenesis*

ETA and the type III cytotoxins play complementary roles toward establishing and maintaining *P. aeruginosa* within the host and ultimately harming the host. Whereas ETA acts at a distance from the site of infection, type III cytotoxins are delivered into host cells by direct contact-mediated injection into the host cell and therefore act locally to the site of infection. ETA is secreted into the extracellular environment (McVay and Hamood, 1995) and transported throughout the host to target tissues and organs. ETA binds to the mammalian cell through the alpha 2-macroglobulin receptor/low-density lipoprotein receptor-related protein, which internalizes ETA via receptor-mediated endocytosis (Kounnas *et al*., 1992). Since the receptor is widely distributed and numerous organs and tissues can be harmed, ETA provides *P. aeruginosa* an advantage by compromising host functions that are beyond the site of colonization. The production of ETA as a proenzyme that is activated at the cell surface by furin (Inocencio *et al*., 1994) provides a mechanism to stabilize the toxin during transport throughout the cell. In contrast, the direct injection of the type III cytotoxins into a host cell provides the immediate ability to compromise macrophages and neutrophils that respond to the initial colonization of the host by *P. aeruginosa*.

#### *Multiple catalytic activities of ExoS and ExoT, analogous to the type III cytotoxins of* Yersinia *and* Salmonella

The discovery that ExoS and ExoT are bifunctional enzymes, as opposed to the more typical situation where a toxin encodes a single enzymatic activity, raises an interesting issue about their role in *P. aeruginosa* pathogenesis, including the coordination, potency, and specificity in the modulation of host cell physiology.

*Coordination: Salmonella* invades mammalian cells by modulating the cell cytoskeleton through the sequential action of two type III cytotoxins. Initially, SopE activates Rho GTPases to promote invasion (Galan, 2001) and subsequently, SptP inactivates Rho GTPases to

reverse actin reorganization required for uptake (Galan, 2001). The two opposing activities are temporally modulated to allow *Salmonella* to effectively invade a mammalian cell where differential stability of the two proteins within the cell allows the invasion phenotype (Kubori and Galan, 2003). In contrast, the expression of two independent catalytic activities within ExoS and ExoT imply the importance of a coordinated expression of Rho GAP and ADP-ribosylation at a similar stage of the infection.

*Potency: Yersinia* utilize multiple cytotoxins to dampen the host immune response to infection, which coordinately inactivate actin organization and downregulate host immune response (Cornelis, 2002a). Although the deletion of one *Yersinia* type III cytotoxin often yields only a limited effect on pathogenesis, deletion of multiple cytotoxins with similar or unique functions can have profound effects on virulence (Logsdon and Mecsas, 2003). Thus, the effective inactivation of host defense mechanisms requires multiple bacterial virulence factors. This may parallel the type III cytotoxins of *P. aeruginosa*, where the expression of RhoGAP and ADP-ribosyltransferase activities function in concert to efficiently inactivate one or more signal transduction pathways. Support of this concept comes from the recent finding that ExoS ADP-ribosylates the ERM proteins during an infection (Maresso and Barbieri, in press). ERM proteins modulate Rho activation by facilitating the removal of Rho GDI from inactive Rho GTPases and potentially complementing RhoGAP activity. Likewise, the recent determination that ExoT ADP-ribosylates the Crk proteins (Sun and Barbieri, 2003) provides a second mechanism for the inactivation of Rac signaling that can augment Rho GAP activity. Inactivation from two different upstream mechanisms would provide potent and efficient inactivation of Rho signal transduction.

*Specificity*: Another role for bifunctionality may be related with the wide range of environments that *P. aeruginosa* inhabits, from soil to a mammalian host. To adapt to this wide range of conditions, *P. aeruginosa* may need multiple, overlapping adaptive mechanisms where the RhoGAP or ADP-ribosyltransferase activities may not have evolved to function within the same environmental niche.

### *Covalent versus non-covalent modification of host cell proteins; analogous, but unique to* **Salmonella**

The discovery of bacterial GAPs, GEFs, and adenylyl cyclases add another dimension to toxin biology: modulation of host cell physiology by non-covalent mechanisms. The acquisition of a non-covalent enzymatic activity by a bacterial pathogen to modulate host cell physiology may allow a framework to modulate the infectious cycle. This strategy is also illustrated in *Salmonella*, with SopE and SptP. In contrast, covalent modifications are permanent, unless a host enzyme can remove the modification. Thus, non-covalent modifications allow the bacterium to temporally control the extent and timing of host cell modification.

## CONCLUSION

Since the initial discoveries of ETA and exoenzyme S, progress has been made in characterizing cytotoxins of *P. aeruginosa*. Their analysis has led to insights into not only toxin biology and virulence mechanisms by pathogenic bacteria, but also the physiology of the mammalian cell. Understanding the function of *P. aeruginosa* cytotoxins may also lead to therapies to control the pathogenesis of this unique and versatile microorganism. Advances in understanding the action of ETA have also led to the development of therapies to cure specific cancers through immunotherapy (FitzGerald *et al*., 2004), while characterization of the type III secretion system may lead to novel strategies for early detection (Banwart *et al*., 2002) and therapies (Frank *et al*., 2002) to control *P. aeruginosa* infections.

## REFERENCES

Adams, C., Morris-Quinn, M., McConnell, F., West, J., Lucey, B., Shortt, C., Cryan, B., Watson, J.B. and O'Gara, F. (1998). Epidemiology and clinical impact of Pseudomonas aeruginosa infection in cystic fibrosis using AP-PCR fingerprinting. *J. Infect*. **37**, 151–158.

Aktories, K., Jung, M., Bohmer, J., Fritz, G., Vandekerckhove, J. and Just, I. (1995). Studies on the active-site structure of C3-like exoenzymes: involvement of glutamic acid in catalysis of ADP-ribosylation. *Biochimie* **77**, 326–332.

Allured, V.S., Collier, R.J., Carroll, S.F. and McKay, D.B. (1986). Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution. *Proc. Natl. Acad. Sci. USA* **83**, 1320–1324.

Banwart, B., Splaingard, M.L., Farrell, P.M., Rock, M.J., Havens, P.L., Moss, J., Ehrmantraut, M.E., Frank, D.W. and Barbieri, J.T. (2002). Children with cystic fibrosis produce an immune response against exoenzyme S, a type III cytotoxin of Pseudomonas aeruginosa. *J. Infect. Dis*. **185**, 269–270.

Barbieri, J.T. (2000). Pseudomonas aeruginosa exoenzyme S, a bifunctional type III secreted cytotoxin. *Int. J. Med. Microbiol*. **290**, 381–387.

Black, D.S. and Bliska, J.B. (2000). The RhoGAP activity of the Yersinia pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence. *Mol. Microbiol*. **37**, 515–527.

Broms, J.E., Sundin, C., Francis, M.S. and Forsberg, A. (2003). Comparative analysis of type III effector translocation by Yersinia pseudotuberculosis expressing native LcrV or PcrV from Pseudomonas aeruginosa. *J. Infect. Dis*. **188**, 239–249.

Carroll, S.F. and Collier, R.J. (1987). Active site of Pseudomonas aeruginosa exotoxin A. Glutamic acid 553 is photolabeled by NAD and shows functional homology with glutamic acid 148 of diphtheria toxin. *J. Biol. Chem*. **262**, 8707–8711.

Carroll, S.F., McCloskey, J.A., Crain, P.F., Oppenheimer, N.J., Marschner, T.M. and Collier, R.J. (1985). Photoaffinity labeling of diphtheria toxin fragment A with NAD: structure of the photoproduct at position 148. *Proc. Natl. Acad. Sci. USA* **82**, 7237–7241.

Carty, N.L., Rumbaugh, K.P. and Hamood, A.N. (2003). Regulation of toxA by PtxR in Pseudomonas aeruginosa PA103. *Can. J. Microbiol*. **49**, 450–464.

Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989). The mammalian G protein rhoC is ADP-ribosylated by Clostridium botulinum exoenzyme C3 and affects actin microfilaments in Vero cells. *EMBO J*. **8**, 1087–1092.

Chaudhary, V.K., Jinno, Y., FitzGerald, D. and Pastan, I. (1990). Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. Natl. Acad. Sci. USA* **87**, 308–312.

Chiron, M.F., Fryling, C.M. and FitzGerald, D.J. (1994). Cleavage of pseudomonas exotoxin and diphtheria toxin by a furin-like enzyme prepared from beef liver. *J. Biol. Chem*. **269**, 18167–18176.

Coburn, J., Dillon, S.T., Iglewski, B.H. and Gill, D.M. (1989a). Exoenzyme S of Pseudomonas aeruginosa ADP-ribosylates the intermediate filament protein vimentin. *Infect. Immun*. **57**, 996–998.

Coburn, J. and Gill, D.M. (1991). ADP-ribosylation of p21ras and related proteins by Pseudomonas aeruginosa exoenzyme S. *Infect. Immun*. **59**, 4259–4262.

Coburn, J., Kane, A.V., Feig, L. and Gill, D.M. (1991). Pseudomonas aeruginosa exoenzyme S requires a eukaryotic protein for ADP-ribosyltransferase activity. *J. Biol. Chem*. **266**, 6438–6446.

Coburn, J., Wyatt, R.T., Iglewski, B.H. and Gill, D.M. (1989b). Several GTP-binding proteins, including p21c-H-ras, are preferred substrates of Pseudomonas aeruginosa exoenzyme S. *J. Biol. Chem*. **264**, 9004–9008.

Cornelis, G.R. (2002a). Yersinia type III secretion: send in the effectors. *J. Cell. Biol*. **158**, 401–408.

Cornelis, G.R. (2002b). The Yersinia Ysc-Yop virulence apparatus. *Int. J. Med. Microbiol*. **291**, 455–462.

Douglas, C.M. and Collier, R.J. (1990). Pseudomonas aeruginosa exotoxin A: alterations of biological and biochemical properties resulting from mutation of glutamic acid 553 to aspartic acid. *Biochemistry* **29**, 5043–5049.

Escuyer, V., Duflot, E., Sezer, O., Danchin, A. and Mock, M. (1988). Structural homology between virulence-associated bacterial adenylate cyclases. *Gene* **71**, 293–298.

Feng, Y., Jadhav, A.P., Rodighiero, C., Fujinaga, Y., Kirchhausen, T. and Lencer, W.I. (2004). Retrograde transport of cholera toxin from the plasma membrane to the endoplasmic reticulum requires the trans-Golgi network but not the Golgi apparatus in Exo2-treated cells. *EMBO Rep* **5**, 596–601.

Finck-Barbancon, V. and Frank, D.W. (2001). Multiple domains are required for the toxic activity of Pseudomonas aeruginosa ExoU. *J. Bacteriol*. **183**, 4330–4344.

Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J.P., Fleiszig, S.M., Wu, C., Mende-Mueller, L. and Frank, D.W. (1997). ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. *Mol. Microbiol*. **25**, 547–557.

Finland, M. (1972). Changing patterns of susceptibility of common bacterial pathogens to antimicrobial agents. *Ann. Intern. Med*. **76**, 1009–1036.

FitzGerald, D.J., Kreitman, R., Wilson, W., Squires, D. and Pastan, I. (2004). Recombinant immunotoxins for treating cancer. *Int. J. Med. Microbiol*. **293**, 577–582.

Fleiszig, S.M., Wiener-Kronish, J.P., Miyazaki, H., Vallas, V., Mostov, K.E., Kanada, D., Sawa, T., Yen, T.S. and Frank, D.W. (1997). Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect. Immun*. **65**, 579–586.

Frank, D.W. (1997). The exoenzyme S regulon of Pseudomonas aeruginosa. *Mol. Microbiol*. **26**, 621–629.

Frank, D.W., Vallis, A., Wiener-Kronish, J.P., Roy-Burman, A., Spack, E.G., Mullaney, B.P., Megdoud, M., Marks, J.D., Fritz, R. and Sawa, T. (2002). Generation and characterization of a protective monoclonal antibody to Pseudomonas aeruginosa PcrV. *J. Infect. Dis*. **186**, 64–73.

Fraylick, J.E., Riese, M.J., Vincent, T.S., Barbieri, J.T. and Olson, J.C. (2002). ADP-ribosylation and functional effects of Pseudomonas exoenzyme S on cellular RalA. *Biochemistry* **41**, 9680–9687.

Frithz-Lindsten, E., Du, Y., Rosqvist, R. and Forsberg, A. (1997). Intracellular targeting of exoenzyme S of Pseudomonas aeruginosa via type III-dependent translocation induces phagocytosis resistance, cytotoxicity, and disruption of actin microfilaments. *Mol. Microbiol*. **25**, 1125–1139.

Frithz-Lindsten, E., Holmstrom, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R. and Forsberg, A. (1998). Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of Pseudomonas aeruginosa and Yersinia pseudotuberculosis. *Mol. Microbiol*. **29**, 1155–1165.

Fu, H., Coburn, J. and Collier, R.J. (1993). The eukaryotic host factor that activates exoenzyme S of Pseudomonas aeruginosa is a member of the 14-3-3 protein family. *Proc. Natl. Acad. Sci. USA* **90**, 2320–2324.

Galan, J.E. (2001). Salmonella interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol*. **17**, 53–86.

Ganesan, A.K., Frank, D.W., Misra, R.P., Schmidt, G. and Barbieri, J.T. (1998). Pseudomonas aeruginosa exoenzyme S ADP-ribosylates Ras at multiple sites. *J. Biol. Chem*. **273**, 7332–7337.

Ganesan, A.K., Mende-Mueller, L., Selzer, J. and Barbieri, J.T. (1999a). Pseudomonas aeruginosa exoenzyme S, a double ADP-ribosyltransferase, resembles vertebrate mono-ADP-ribosyltransferases. *J. Biol. Chem*. **274**, 9503–9508.

Ganesan, A.K., Vincent, T.S., Olson, J.C. and Barbieri, J.T. (1999b). Pseudomonas aeruginosa exoenzyme S disrupts Ras-mediated signal transduction by inhibiting guanine nucleotide exchange factor-catalyzed nucleotide exchange. *J. Biol. Chem*. **274**, 21823–21829.

Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A. and Engel, J.N. (2000). The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of Pseudomonas aeruginosa by epithelial cells and macrophages. *Infect. Immun*. **68**, 7100–7113.

Geiser, T.K., Kazmierczak, B.I., Garrity-Ryan, L.K., Matthay, M.A. and Engel, J.N. (2001). Pseudomonas aeruginosa ExoT inhibits *in vitro* lung epithelial wound repair. *Cell Microbiol*. **3**, 223–236.

Glaser, P., Munier, H., Gilles, A.M., Krin, E., Porumb, T., Barzu, O., Sarfati, R., Pellecuer, C. and Danchin, A. (1991). Functional consequences of single amino acid substitutions in calmodulin-activated adenylate cyclase of Bordetella pertussis. *EMBO J*. **10**, 1683–1688.

Goehring, U.M., Schmidt, G., Pederson, K.J., Aktories, K. and Barbieri, J.T. (1999). The N-terminal domain of Pseudomonas aeruginosa exoenzyme S is a GTPase-activating protein for Rho GTPases. *J. Biol. Chem*. **274**, 36369–36372.

Gray, G.L., Smith, D.H., Baldridge, J.S., Harkins, R.N., Vasil, M.L., Chen, E.Y. and Heyneker, H.L. (1984). Cloning, nucleotide sequence, and expression in Escherichia coli of the exotoxin A structural gene of Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. USA*. **81**, 2645–2649.

Hallberg, B. (2002). Exoenzyme S binds its cofactor 14-3-3 through a non-phosphorylated motif. *Biochem. Soc. Trans*. **30**, 401–405.

Han, S., Arvai, A.S., Clancy, S.B. and Tainer, J.A. (2001). Crystal structure and novel recognition motif of rho ADP-ribosylating C3 exoenzyme from Clostridium botulinum: structural insights for recognition specificity and catalysis. *J. Mol. Biol*. **305**, 95–107.

Han, S., Craig, J.A., Putnam, C.D., Carozzi, N.B. and Tainer, J.A. (1999). Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. *Nat. Struct. Biol*. **6**, 932–936.

Hauser, A.R., Kang, P.J. and Engel, J.N. (1998). PepA, a secreted protein of Pseudomonas aeruginosa, is necessary for cytotoxicity and virulence. *Mol. Microbiol*. **27**, 807–818.

Henriksson, M.L., Sundin, C., Jansson, A.L., Forsberg, A., Palmer, R.H. and Hallberg, B. (2002). Exosenzyme S show selective ADP-ribosylation and GAP activities towards small GTPases *in vivo*. *Biochem. J. Pt*.

Hwang, J., Fitzgerald, D.J., Adhya, S. and Pastan, I. (1987). Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell* **48**, 129–136.

Iglewski, B.H., Liu, P.V. and Kabat, D. (1977). Mechanism of action of Pseudomonas aeruginosa exotoxin Aiadenosine diphosphate-ribosylation of mammalian elongation factor 2 *in vitro* and *in vivo*. *Infect. Immun*. **15**, 138–144.

Iglewski, B.H., Sadoff, J., Bjorn, M.J. and Maxwell, E.S. (1978). Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. *Proc. Natl. Acad. Sci. USA* **75**, 3211–3215.

Inocencio, N.M., Moehring, J.M. and Moehring, T.J. (1994). Furin activates Pseudomonas exotoxin A by specific cleavage *in vivo* and *in vitro*. *J. Biol. Chem*. **269**, 31831–31835.

Kang, P.J., Hauser, A.R., Apodaca, G., Fleiszig, S.M., Wiener-Kronish, J., Mostov, K. and Engel, J.N. (1997). Identification of Pseudomonas aeruginosa genes required for epithelial cell injury. *Mol. Microbiol*. **24**, 1249–1262.

Kazama, H., Hamashima, H., Sasatsu, M. and Arai, T. (1998). Distribution of the antiseptic-resistance genes qacE and qacE delta 1 in Gram-negative bacteria. *FEMS Microbiol. Lett*. **159**, 173–178.

Knight, D.A. and Barbieri, J.T. (1997). Ecto-ADP-ribosyltransferase activity of Pseudomonas aeruginosa exoenzyme S. *Infect. Immun*. **65**, 3304–3309.

Knight, D.A. and Barbieri, J.T. (1999). Expression of FAS-independent ADP-ribosyltransferase activity by a catalytic deletion peptide of Pseudomonas aeruginosa exoenzyme S. *Biochemistry* **38**, 5858–5863.

Knight, D.A., Finck-Barbancon, V., Kulich, S.M. and Barbieri, J.T. (1995). Functional domains of Pseudomonas aeruginosa exoenzyme S. *Infect. Immun*. **63**, 3182–3186.

Kounnas, M.Z., Morris, R.E., Thompson, M.R., FitzGerald, D.J., Strickland, D.K. and Saelinger, C.B. (1992). The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem*. **267**, 12420–12423.

Krall, R., Schmidt, G., Aktories, K. and Barbieri, J.T. (2000). Pseudomonas aeruginosa ExoT is a Rho GTPase-activating protein. *Infect. Immun*. **68**, 6066–6068.

Krall, R., Sun, J., Pederson, K.J. and Barbieri, J.T. (2002). *In vivo* Rho GTPase-activating protein activity of Pseudomonas aeruginosa cytotoxin ExoS. *Infect. Immun*. **70**, 360–367.

Kubori, T. and Galan, J.E. (2003). Temporal regulation of salmonella virulence effector function by proteasome-dependent protein degradation. *Cell* **115**, 333–342.

Kulich, S.M., Frank, D.W. and Barbieri, J.T. (1993). Purification and characterization of exoenzyme S from Pseudomonas aeruginosa 388. *Infect. Immun*. **61**, 307–313.

Kulich, S.M., Yahr, T.L., Mende-Mueller, L.M., Barbieri, J.T. and Frank, D.W. (1994). Cloning the structural gene for the 49-kDa form of exoenzyme S (exoS) from Pseudomonas aeruginosa strain 388. *J. Biol. Chem*. **269**, 10431–10437.

Lee, H. and Iglewski, W.J. (1984). Cellular ADP-ribosyltransferase with the same mechanism of action as diphtheria toxin and Pseudomonas toxin A. *Proc. Natl. Acad. Sci. USA* **81**, 2703–2707.

Liu, S., Kulich, S.M. and Barbieri, J.T. (1996). Identification of glutamic acid 381 as a candidate active site residue of Pseudomonas aeruginosa exoenzyme S. *Biochemistry* **35**, 2754–2758.

Liu, S., Yahr, T.L., Frank, D.W. and Barbieri, J.T. (1997). Biochemical relationships between the 53-kilodalton (Exo53) and 49-kilodalton (ExoS) forms of exoenzyme S of Pseudomonas aeruginosa. *J. Bacteriol*. **179**, 1609–1613.

Logsdon, L.K. and Mecsas, J. (2003). Requirement of the Yersinia pseudotuberculosis effectors YopH and YopE in colonization and persistence in intestinal and lymph tissues. *Infect. Immun*. **71**, 4595–4607.

Mangeat, P., Roy, C. and Martin, M. (1999). ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol*. **9**, 187–192.

Maresso, A.W., Baldwin, M.R. and Barbieri, J.T. (2004). Ezrin/radixin/moesin proteins are high affinity targets for ADP-ribosylation by Pseudomonas aeruginosa ExoS. *J. Biol. Chem*. **279**, 38402–38408.

Maresso, A.W. and Barbieri, J.T. (2002). Expression and purification of two recombinant forms of the type-III cytotoxin, Pseudomonas aeruginosa ExoS. *Protein Expr. Purif*. **26**, 432–437.

Maresso, A.W., Riese, M.J. and Barbieri, J.T. (2003). Molecular heterogeneity of a type III cytotoxin, Pseudomonas aeruginosa exoenzyme S. *Biochemistry* **42**, 14249–14257.

Masters, S.C., Pederson, K.J., Zhang, L., Barbieri, J.T. and Fu, H. (1999). Interaction of 14-3-3 with a nonphosphorylated protein ligand, exoenzyme S of Pseudomonas aeruginosa. *Biochemistry* **38**, 5216–5221.

McGuffie, E.M., Frank, D.W., Vincent, T.S. and Olson, J.C. (1998). Modification of Ras in eukaryotic cells by Pseudomonas aeruginosa exoenzyme S. *Infect. Immun*. **66**, 2607–2613.

McVay, C.S. and Hamood, A.N. (1995). Toxin A secretion in Pseudomonas aeruginosa: the role of the first 30 amino acids of the mature toxin. *Mol. Gen. Genet*. **249**, 515–525.

Mendelson, M.H., Gurtman, A., Szabo, S., Neibart, E., Meyers, B.R., Policar, M., Cheung, T.W., Lillienfeld, D., Hammer, G., Reddy, S. and *et al*. (1994). Pseudomonas aeruginosa bacteremia in patients with AIDS. *Clin. Infect. Dis*. **18**, 886–895.

Mock, M., Labruyere, E., Glaser, P., Danchin, A. and Ullmann, A. (1988). Cloning and expression of the calmodulin-sensitive Bacillus anthracis adenylate cyclase in Escherichia coli. *Gene* **64**, 277–284.

Mohr, C., Koch, G., Just, I. and Aktories, K. (1992). ADP-ribosylation by Clostridium botulinum C3 exoenzyme increases steady-state GTPase activities of recombinant rhoA and rhoB proteins. *FEBS Lett*. **297**, 95–99.

Nalefski, E.A., Sultzman, L.A., Martin, D.M., Kriz, R.W., Towler, P.S., Knopf, J.L. and Clark, J.D. (1994). Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. *J. Biol. Chem*. **269**, 18239–18249.

Nicas, T.I., Bradley, J., Lochner, J.E. and Iglewski, B.H. (1985). The role of exoenzyme S in infections with Pseudomonas aeruginosa. *J. Infect. Dis*. **152**, 716–721.

Nicas, T.I. and Iglewski, B.H. (1984). Isolation and characterization of transposon-induced mutants of Pseudomonas aeruginosa deficient in production of exoenzyme S. *Infect. Immun*. **45**, 470–474.

Nicas, T.I. and Iglewski, B.H. (1985). The contribution of exoproducts to virulence of Pseudomonas aeruginosa. *Can. J. Microbiol*. **31**, 387–392.

Ogata, M., Chaudhary, V.K., Pastan, I. and FitzGerald, D.J. (1990). Processing of Pseudomonas exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J. Biol. Chem*. **265**, 20678–20685.

Ogata, M., Fryling, C.M., Pastan, I. and FitzGerald, D.J. (1992). Cell-mediated cleavage of Pseudomonas exotoxin between Arg279 and Gly280 generates the enzymatically active fragment, which translocates to the cytosol. *J. Biol. Chem*. **267**, 25396–25401.

Okazaki, I.J. and Moss, J. (1996). Structure and function of eukaryotic mono-ADP-ribosyltransferases. *Rev. Physiol. Biochem. Pharmacol*. **129**, 51–104.

Pederson, K.J. and Barbieri, J.T. (1998). Intracellular expression of the ADP-ribosyltransferase domain of Pseudomonas exoenzyme S is cytotoxic to eukaryotic cells. *Mol. Microbiol*. **30**, 751–759.

Pederson, K.J., Krall, R., Riese, M.J. and Barbieri, J.T. (2002). Intracellular localization modulates targeting of ExoS, a type III cytotoxin, to eukaryotic signaling proteins. *Mol. Microbiol*. **46**, 1381–1390.

Pederson, K.J., Vallis, A.J., Aktories, K., Frank, D.W. and Barbieri, J.T. (1999). The amino-terminal domain of Pseudomonas aeruginosa ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. *Mol. Microbiol*. **32**, 393–401.

Phillips, R.M., Six, D.A., Dennis, E.A. and Ghosh, P. (2003). *In vivo* phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. *J. Biol. Chem*. **278**, 41326–41332.

Radke, J., Pederson, K.J. and Barbieri, J.T. (1999). Pseudomonas aeruginosa exoenzyme S is a biglutamic acid ADP-ribosyltransferase. *Infect. Immun*. **67**, 1508–1510.

Riese, M.J. and Barbieri, J.T. (2002). Membrane localization contributes to the *in vivo* ADP-ribosylation of Ras by Pseudomonas aeruginosa ExoS. *Infect. Immun*. **70**, 2230–2232.

Riese, M.J., Goehring, U.M., Ehrmantraut, M.E., Moss, J., Barbieri, J.T., Aktories, K. and Schmidt, G. (2002). Auto-ADP-ribosylation of Pseudomonas aeruginosa ExoS. *J. Biol. Chem*. **277**, 12082–12088.

Riese, M.J., Wittinghofer, A. and Barbieri, J.T. (2001). ADP-ribosylation of Arg41 of Rap by ExoS inhibits the ability of Rap to interact with its guanine nucleotide exchange factor, C3G. *Biochemistry* **40**, 3289–3294.

Sato, H. and Frank, D.W. (2004). ExoU is a potent intracellular phospholipase. *Mol. Microbiol*. **53**, 1279–1290.

Sato, H., Frank, D.W., Hillard, C.J., Feix, J.B., Pankhaniya, R.R., Moriyama, K., Finck-Barbancon, V., Buchaklian, A., Lei, M., Long, R.M., Wiener-Kronish, J. and Sawa, T. (2003). The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. *EMBO J*. **22**, 2959–2969.

Sato, H., J. B. Feix, C. J. Hillard and D. W. Frank. (2005). The characterization of phospholipase activity of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. *J. Bacteriol*. In press.

Sawa, T., Yahr, T.L., Ohara, M., Kurahashi, K., Gropper, M.A., Wiener-Kronish, J.P. and Frank, D.W. (1999). Active and passive immunization with the Pseudomonas V antigen protects against type III intoxication and lung injury. *Nat. Med*. **5**, 392–398.

Sayner, S.L., Frank, D.W., King, J., Chen, H., VandeWaa, J. and Stevens, T. (2004). Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by Pseudomonas aeruginosa ExoY. *Circ. Res*. **95**, 196–203.

Sun, J. and Barbieri, J.T. (2003). Pseudomonas aeruginosa ExoT ADP-ribosylates CT10-regulator of kinase (Crk) proteins. *J. Biol. Chem*.

Sun, J., Maresso, A.W., Kim, J.J. and Barbieri, J.T. (2004). How bacterial ADP-ribosylating toxins recognize substrates. *Nat Struct Mol Biol.*

Sundin, C., Henriksson, M.L., Hallberg, B., Forsberg, A. and Frithz-Lindsten, E. (2001). Exoenzyme T of Pseudomonas aeruginosa elicits cytotoxicity without interfering with Ras signal transduction. *Cell Microbiol*. **3**, 237–246.

Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matuoka, K., Takenawa, T., Kurata, T., Nagashima, K. *et al*. (1994). C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. *Proc. Natl. Acad. Sci. USA* **91**, 3443–3447.

Tsuge, H., Nagahama, M., Nishimura, H., Hisatsune, J., Sakaguchi, Y., Itogawa, Y., Katunuma, N. and Sakurai, J. (2003). Crystal structure and site-directed mutagenesis of enzymatic components from Clostridium perfringens iota-toxin. *J. Mol. Biol*. **325**, 471–483.

Van Delden, C. and Iglewski, B.H. (1998). Cell-to-cell signaling and Pseudomonas aeruginosa infections. *Emerg. Infect. Dis*. **4**, 551–560.

Wedekind, J.E., Trame, C.B., Dorywalska, M., Koehl, P., Raschke, T.M., McKee, M., FitzGerald, D., Collier, R.J. and McKay, D.B. (2001). Refined crystallographic structure of Pseudomonas aeruginosa exotoxin A and its implications for the molecular mechanism of toxicity. *J. Mol. Biol*. **314**, 823–837.

Wiesmuller, L. and Wittinghofer, F. (1994). Signal transduction pathways involving Ras. Mini review. *Cell Signal* **6**, 247–267.

Wilde, C., Just, I. and Aktories, K. (2002). Structure-function analysis of the Rho-ADP-ribosylating exoenzyme C3stau2 from Staphylococcus aureus. *Biochemistry* **41**, 1539–1544.

Willer, M., Jermy, A.J., Young, B.P. and Stirling, C.J. (2003). Identification of novel protein-protein interactions at the cytosolic surface of the Sec63 complex in the yeast ER membrane. *Yeast* **20**, 133–148.

Wurtele, M., Wolf, E., Pederson, K.J., Buchwald, G., Ahmadian, M.R., Barbieri, J.T. and Wittinghofer, A. (2001). How the Pseudomonas aeruginosa ExoS toxin down-regulates Rac. *Nat. Struct. Biol*. **8**, 23–26.

Yahr, T.L., Mende-Mueller, L.M., Friese, M.B. and Frank, D.W. (1997). Identification of type III secreted products of the Pseudomonas aeruginosa exoenzyme S regulon. *J. Bacteriol*. **179**, 7165–7168.

Yahr, T.L., Vallis, A.J., Hancock, M.K., Barbieri, J.T. and Frank, D.W. (1998). ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system. *Proc. Natl. Acad. Sci. USA* **95**, 13899–13904.

CHAPTER

# 15

# *Vibrio cholerae* and *Escherichia coli* thermolabile enterotoxin

*Timothy R. Hirst and Jocelyne M. D'Souza*

## INTRODUCTION

Historical accounts of cholera-like disease date back to the times of Hippocrates and Buddha (Barua, 1991), and today the devastating impact of cholera is still evident in developing countries. Cholera toxin (Ctx) from *Vibrio cholerae*, and subsequently the closely related heat-labile enterotoxins (Etx) from certain toxigenic strains of *Escherichia coli* were among the earliest bacterial toxins to be characterized. In the past 25 years there has been a growing appreciation that the cholera toxin family of thermolabile enterotoxins has provided an inspirational insight into some of the great themes of microbial pathogenicity. For example, studies on the biogenesis of Ctx in *V. cholerae* have provided important insights into the mechanisms of gene regulation and protein secretion in bacteria, and resulted in Ctx being a paradigm of virulence factor expression. Studies on the interaction of Ctx and Etx in epithelial cells have illuminated the mechanisms by which the toxins cause severe, and at times, life-threatening disease. Moreover, they have provided considerable insight into the cellular events associated with vesicular movement and targeting, a central feature of eukaryotic cell biology.

A scant survey of the number of papers citing cholera toxin that are listed by PubMed reveals that number will soon pass 10,000. This startlingly high number reflects the magnitude of research into these molecules, as well as the widespread use of cholera toxin as a biochemical reagent to elevate cAMP in mammalian cells, and to its ever growing use as a vaccine adjuvant. Such applications and uses of cholera toxin and *E. coli* thermolabile enterotoxins are not covered in this chapter; it instead focuses on the remarkable "life and times" of these toxins, from the genetics of toxin expression, assembly, and secretion in the bacteria, to their interaction, uptake, and action in disease.

## CHOLERA AND RELATED ENTEROPATHIES

Robert Koch, the great German bacteriologist, established that a "comma-bacillus," now called *V. cholerae* serogroup O1, was the causative agent of Asiatic cholera (Koch, 1884). The organism, which is transmitted in contaminated water, colonizes the surface of the small bowel and induces the production of copious quantities of watery diarrhea. The rapid spread of the organism and its capacity to reduce a previously healthy individual to the point of death in as little as 6–8 hours made cholera a disease to be feared. However, with the widespread introduction of fluid and electrolyte replacement therapies (intravenous or oral), the mortality rate can be reduced to less than 1%.

Nonetheless, the devastating impact of cholera is all too evident, as seen in the Rwandan refugee camps in 1994 when an estimated 600,000 cases of cholera occurred over a three-week period, including 45,000 deaths (Waldman, 1998). The worldwide incidence of cholera is difficult to evaluate, although it has been estimated that there are approximately 8 million cases per annum, including 124,000 deaths (Black, 1986). In

1992, a new serogroup of *V. cholerae* emerged that caused a large number of cholera cases and deaths in India and Bangladesh alone during the first three months of 1993 (Albert *et al.*, 1993; Sack *et al.*, 1996). The strain did not react with antibodies against the O1-antigen or any of the other 137 non-O1 serogroups and has been designated O139. Although *V. cholerae* O139 produces a different O-antigen, it shares many pathogenic features with *V. cholerae* O1 (biotype El Tor), including production of Ctx (Nakashima *et al.*, 1995).

In 1956, S.N. De noted that the common gut bacterium *E. coli* was also capable of producing a cholera-like disease (De *et al.*, 1956). By the early 1970s, it was clear that enterotoxigenic *E. coli* (ETEC) were of global significance as agents of diarrheal disease in both humans and domestic animals. ETEC have a worldwide distribution, but their significance as human pathogens is most evident in developing countries, where it has been estimated that approximately 20% of all acute (life-threatening) diarrheal cases in children under 5 years of age are due to ETEC (Evans *et al.*, 1977; Black *et al.*, 1980; Agbonlahor and Odugbemi, 1982; de Mol *et al.*, 1983; Echeverria *et al.*, 1985; Steffen, 1986). The secretory diarrhea produced by ETEC is due to its ability to produce either an oligomeric heat-labile enterotoxin (Etx) that is structurally and functionally homologous to Ctx (see below) or a heat-stable enterotoxin (ST) or both (Nataro and Kaper, 1998).

## STRUCTURE OF CHOLERA TOXIN AND RELATED ENTEROTOXINS

Purification and structural analysis of cholera toxin (Ctx) from sterile culture filtrates of *V. cholerae* was first attempted in 1966 (Finkelstein *et al.*, 1966), but it was not until the successful crystallization and X-ray analyses of Ctx almost 30 years later that the toxin arrangement of six non-covalently associated subunits was confirmed (Zhang *et al.*, 1995). Ctx is now well-characterized as a hexameric protein toxin comprised of a single A-subunit (240 amino acids with a molecular weight of 27,500 Da) arranged on a toroidal ring of five B-subunits (103 amino acids each with a molecular weight of 11,800 Da). When Ctx was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, the A-subunit migrated as a single polypeptide with an apparent electrophoretic mobility of *about* 28,000 Da, but under reducing conditions, separated into two polypeptides, A1 ($M_r$ = ~22,000) and A2 ($M_r$ = ~5500), corresponding to the N-terminal and C-terminal fragments of the A-subunit, respectively (Gill, 1975).

### Primary sequence

The primary amino acid sequences of the A- and B-subunits of Etx derived from ETEC strains isolated from humans (hEtx), pigs (pEtx), chickens (cEtx), and Ctx (derived from classical and *El Tor* strains of *V. cholerae* O1 and *V. cholerae* O139) have been deduced (Dallas and Falkow, 1980; Gennaro *et al.*, 1982; Spicer and Noble, 1982; Dallas, 1983; Gennaro and Greenaway, 1983; Lockman and Kaper, 1983; Mekalanos *et al.*, 1983; Yamamoto *et al.*, 1984; Yamamoto *et al.*, 1987; Leong *et al.*, 1985; Takao *et al.*, 1985; Sanchez and Holmgren, 1989; Brickman *et al.*, 1990; Dams *et al.*, 1991; Inoue *et al.*, 1993).

Figure 15.1 shows a comparison of the complete amino acid sequences of the A- and B-subunits of Ctx from the classical *V. cholerae* O1 strain 569B and those of hEtx from an *E. coli* strain, H74-114, of human origin (Leong *et al.*, 1985; Dams *et al.*, 1991). From the sequence, it was apparent that both the A- and B-subunits of Ctx and Etx are synthesized as longer precursors with amino-terminal signal sequence extensions of 18 and 21 amino acids, respectively (denoted by negative numbers in Figure 15.1). Such signal sequences are involved in targeting precursors to the cytoplasmic membrane and are cleaved off during translocation to yield a mature polypeptide.

The mature A-subunits of Ctx (CtxA) and Etx (EtxA), shown in Figure 15.1, are 240 amino acids in length and share considerable sequence identity (82.1%). Similarly, the mature B-subunits (CtxB and EtxB) are comprised of 103 amino acids and share 83.5% identity. The only region where there is little homology between Etx and Ctx is between amino acid residues 190 and 212 of the A-subunits, where the sequence identity is 34.8%. This region contains an exposed disulfide bond between two cysteine residues at positions 187–199. The loop can be easily "nicked" by exogenous proteases, thereby cleaving the A-subunit to its A1/A2 polypeptides and activating the toxin. Commercially available preparations of Ctx contain A-subunits that have already undergone proteolytic nicking, with the cleavage occurring between residues 193 and 193 (Xia *et al.*, 1984). In contrast, Etx purified from *E. coli* is unnicked, although the A-subunit can be easily cleaved by exogenously added trypsin, yielding A1 and A2 polypeptides similar to those of Ctx (Clements and Finkelstein, 1979; Clements *et al.*, 1980). Nicking of the polypeptide chain and reduction of the disulfide bond occurs over 20Å away from the proposed Ctx/Etx active site (van den Akker *et al.*, 1995), suggesting that several key residues distant from the active side might be crucial for the activation pathway. Structural studies of A-subunit mutants of Ctx and Etx have provided insight on the activation and catalytic activity of toxins.

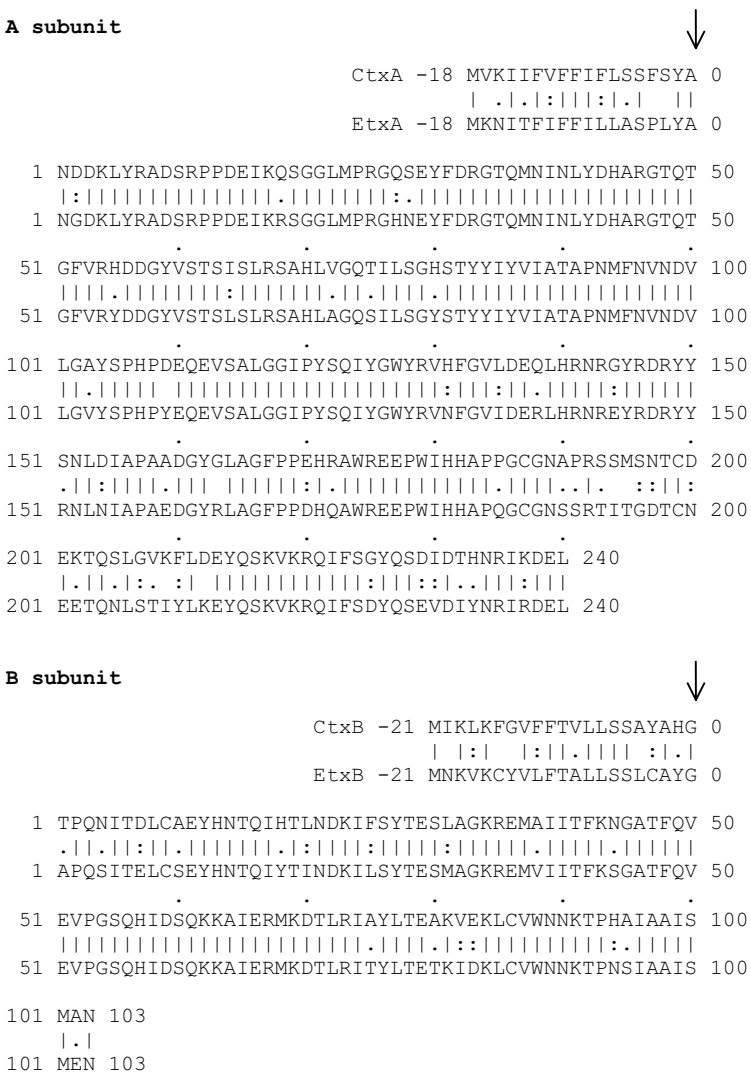



**FIGURE 15.1** Primary amino acid sequence alignments of the A- and B-subunits of cholera toxin and *E. coli* heat-labile enterotoxin. Cholera toxin sequences are from the classical biotype *V. cholerae* O1 strain 569B (Dams *et al.*, 1991); heat-labile enterotoxin sequences are derived from *E. coli* strain H74-114 of human origin (Leong *et al.*, 1985). Amino acids comprising the signal sequences of A- and B-subunits are denoted by negative numbers. The arrows indicate the positions of cleavage between the signal sequences and the mature polypeptides.

Mutagenesis experiments and crystal structure analysis of active Ctx mutants suggest that disorder in the activation loop is necessary and sufficient for toxin activity. This activation loop disorder causes an increase in flexibility of the A-subunit, exposing the active site residues upon substrate binding, thereby rendering the toxin active (O'Neal *et al.*, 2004).

The primary sequences of B-subunits of Ctx and Etx reveal a number of other distinctive features. First, there is a pair of conserved cysteine residues (at positions 9 and 86), which were subsequently shown to form a single intramolecular disulfide bridge (Hardy *et al.*, 1988; Sixma *et al.*, 1991), the formation of which is essential for toxin assembly (see below). Second, they have a high content of charged resides, which for each B-subunit of Ctx includes 3 Asp, 7 Glu, 9 Lys, and 3 Arg residues (Figure 15.1). Third, each B-subunit possesses a single conserved tryptophan residue (at position 88), which forms the floor of the B-subunit's receptor binding function.

Analyses of a variety of ctx genes from different strains of *V. cholerae* have revealed a small number of amino acid differences in the B-subunit sequences compared with that shown in Figure 15.1, and also in the subunits of Etx from human and porcine origin (Tables 15.1 and 15.2). These minor differences have no known effect on the overall 3D structure of the toxin, but they do appear to give rise to significant novel antigenic specificities, and they may influence subtle differences in the receptor binding activities of the toxins.

## Quaternary structure

The successful determination of the X-ray structure of porcine heat-labile enterotoxin from *E. coli* by W.G.J Hol and co-workers in 1991 provided an exquisite

**TABLE 15.1** Amino acid sequence heterogeneity in B-subunits of cholera toxin

| | | Amino acid number[a] B-subunit | | | |
|---|---|---|---|---|---|
| *V. cholerae* strains | Biotype | +18 | +47 | +54 | Reference |
| 569B | Classical | His | Thr | Gly | Dams *et al.* (1991) |
| 41 | Classical | His | Thr | Gly | Dams *et al.* (1991) |
| O395 | Classical | His | Thr | Gly | Dams *et al.* (1991) |
| 2125 | El Tor | Tyr | Ile | Gly | Dams *et al.* (1991) |
| 62746 | El Tor | Tyr | Ile | Gly | Dams *et al.* (1991) |
| 3083 | El Tor | Tyr | Ile | (Ser) | Dams *et al.* (1991) Brickman *et al.* (1990) |

[a]Corresponds to the amino acid number in the mature subunit sequence

insight into the tertiary and quaternary structure of this toxin family (Sixma *et al.*, 1991; Sixma *et al.*, 1993).

This revealed that the A1-fragment had a single-domain structure with a triangular shape, with one side abutting a 23 residue alpha-helix formed by the A2-polypeptide (Figure 15.2). The C-terminal segment of the A2-polypeptide was also revealed to extend downwards into the central pore of the doughnut-shaped ring of five B-subunits (Figure 15.2). Each B-subunit in the pentameric structure is folded into two three-stranded anti-parallel beta-sheets (with one sheet on each side of the monomer facing an adjacent B-subunit) and a large central helix positioned at the wall of the central pore, with the five helices (one from each subunit) forming a pentagonal helix barrel. This forms a pore, 30Å long, with a diameter ranging from ~11Å near the surface at which the A-subunit is positioned to ~15Å on the lower surface. The three-stranded anti-parallel beta-sheets on each side of the B-subunit interact directly with the beta-sheets of the adjacent B-subunits (via multiple hydrogen bonds)—a feature that contributes to the remarkable stability of the B-pentamer. The B-subunit receptor binding site is found on the lower convoluted surface, with each B-monomer possessing a single receptor-site (Hol, 1995; Sixma *et al.*, 1992).

The structural fold of EtxB has also been found in other proteins: staphylococcal nuclease, the anticodon

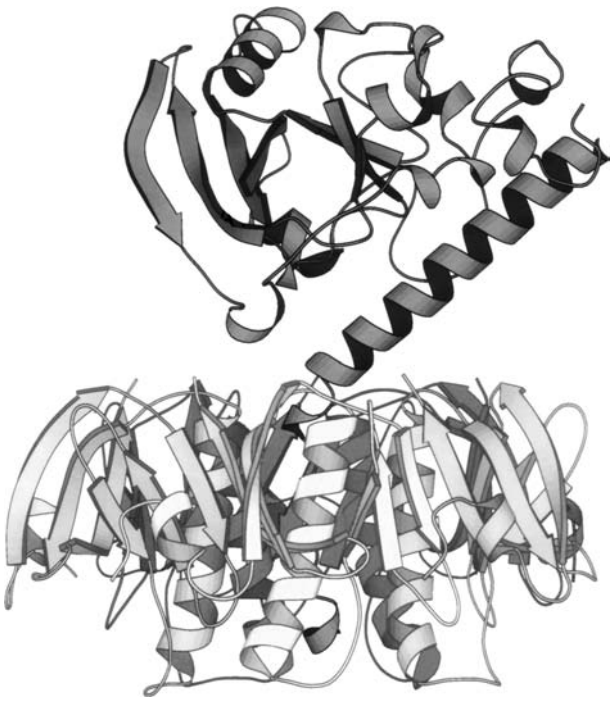


**FIGURE 15.2** Ribbon diagram of the crystal structure of the porcine variant of the *E. coli* heat-labile enterotoxin. The A-subunit (comprised of the A1- and A2-fragments) forms a triangular-shaped structure; the long α-helix of the A2-fragment is clearly visible to the right-hand side. The B-subunits form a pentameric ring with a central pore in which the C-terminal portion of the A2-fragment is situated. The GM1-receptor binding sites in the B-pentamer are located on the lower convoluted side of the molecule.

**TABLE 15.2** Amino acid sequence heterogeneity in A- and B-subunits of Etx from *E. coli* of human and porcine origin

| | | Amino acid number[a] A-subunit | | | | |
|---|---|---|---|---|---|---|
| *E. coli* strain | Origin | +4 | +212 | +213 | +238 | Reference[b] |
| H74-114 | Human | Lys | Lys | Glu | Asp | 1 |
| H10407[c] | Human | Lys | Arg | Lys | Asn | 2,3 |
| P307 | Porcine | Arg | Arg | Glu | Asp | 4 |
| | | **B-subunit** | | | | |
| | | +4 | +13 | +46 | +102 | |
| H74-114 | Human | Ser | His | Ala | Glu | 5 |
| H10407[c] | Human | Ser | Arg | Ala | Glu | 3,6 |
| P307 | Porcine | Thr | Arg | Glu | Lys | 5 |

[a]Corresponds to the amino acid number in the mature subunit sequence

[b]1, Webb and Hirst, unpublished results;
2, Yamamoto *et al.* (1984)
3, Inoue *et al.* (1993)
4, Dykes *et al.* (1985)
5, Leong *et al.* (1985)
6, Yamamoto and Yokota (1983)

[c]The amino acid sequences of the A- and B-subunits of cEtx from chicken ETEC isolate were found to be identical to those of strain H10407 (Inoue *et al.*, 1993).

binding domain of asp-tRNA synthase, which binds oligonucleotides, and verotoxin B-subunit pentamer, which binds a different glycolipid receptor, Gb3 (Arnone *et al.*, 1971; Murzin, 1993; Sixma *et al.*, 1993). The similarity of the B-subunits of Etx (and Ctx) to these other oligosaccharide and oligonucleotide binding proteins has led to the coining of the term "OB-fold" for proteins that possess this overall structure, even though they do not share any homology to their primary sequences.

The porcine variant of Etx used in the crystallographic determination was purified from a recombinant *E. coli* K-12 strain and, as a consequence, the A-subunit was unnicked. The residues 189–195 corresponding to the exposed loop and nicking site between the A1- and A2-polpeptides were not revealed in the electron density map, presumably because of the flexibility of this region. Likewise, the three amino-terminal and four carboxy-terminal amino acids of the A-subunit were not visible within the refined structure.

The subsequent X-ray crystallographic determination of the Ctx structure by Westbrook and co-workers revealed a molecule with a similar overall fold to that of pEtx. The only region of major structural dissimilarity was found in the A2-segment from residue 227, which in Ctx appears to enter the central pore of the B pentamer as an alpha-helix rather than as an extended chain (Zhang *et al.*, 1995). In addition, the last four residues (KDEL) were clearly visibly in the electron density of Ctx and may reflect the more compact structure of the CtxA2- when compared with the EtxA2-fragment. However, a recent determination of the Ctx crystal structure has revealed an elongated conformation of the CtxA2-fragment (O'Neal *et al.*, 2004) in contrast to the helical-type tail proposed by the original published structure of wild-type Ctx (Zhang *et al.*, 1995). The KDEL sequence is disordered in the new Ctx structures, and the observed elongated tail conformation positions this sequence to extend into solvent at the exit of the $B_5$ pore, thus facilitating its proposed interaction with the KDEL receptor (O'Neal *et al.*, 2004).

The A1-polypeptide (amino acids 1–194) contains the enzyme active site capable of ADP-ribosylation (for a fuller description see below). When the crystal structure of Etx was compared with that of another ADP-ribosylating toxin, exotoxin A (ETA) from *Pseudomonas aeruginosa*, it was apparent that 44 residues in the A1-polypeptide could be superimposed with residues in the enzymic domain of ETA (Sixma *et al.*, 1993). Of these, only three amino acids were identical: Tyr 6, Ala 69, and Glu 102 in Etx, with the latter corresponding to the active site residue Glu 533 of ETA.

## BIOGENESIS OF CHOLERA TOXIN AND RELATED ENTEROTOXINS

### Toxin gene organization

One of the important discoveries in cholera research in the past few years was made by Waldor and Mekalanos, who found that the structural genes *ctxA* and *ctxB* encoding the A- and B-subunits of Ctx are located on a single-stranded filamentous phage, CTXΦ (Waldor and Mekalanos, 1996).

The *ctxAB* genes are organized as an operon located near one end of the phage genome (Figure 15.3). The phage contains two regions, a 4.6-kb central "core" and a 2.4-kb repetitive sequence "RS2." The core region of the CTXΦ is composed of the Ctx operon (*ctxAB*) and four additional genes, namely *zot* (encoding the zonula occludes toxin), *ace* (encoding the accessory cholera enterotoxin), *cep* (encoding a core-encoded pilin), and *orfU* (encoding the M13 gene III homologue), all of which have homology to gene sequences from the filamentous bacteriophage M13 (Mekalanos, 1983; Mekalanos *et al.*, 1983; Goldberg and Mekalanos, 1986; Fasano *et al.*, 1991; Baudry *et al.*, 1992; Pearson *et al.*, 1993; Trucksis *et al.*, 1993; Waldor *et al.*, 1997). These genes along with *psh* (from RS2) are required for phage packaging and morphogenesis.

Toxigenic *V. cholerae* also contain a 2.7-kb CTXΦ-related element "RS1" adjacent to the CTX prophage. The DNA sequences of RS1 and RS2 are almost identical, and both encode proteins required for replication (RstA), integration (RstB), and regulatory gene expression (RstR) (Waldor *et al.*, 1997). In addition, RS1 encodes a novel protein, RstC, an antirepressor that promotes the expression of CTXΦ genes, resulting in the transmission of CTXΦ and production of Ctx (Davis *et al.*, 2002). RS1 was recently shown to be a "satellite" phage of CTXΦ capable of propagating horizontally as a filamentous phage (Davis *et al.*, 2002; Faruque *et al.*, 2002).

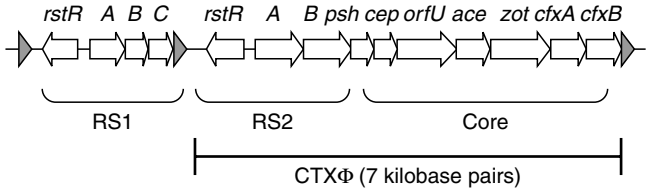



**FIGURE 15.3** Schematic representation of the genetic organization of the CTX DNA element. The 7.0-kb CTXΦ element is comprised of a 2.5-kb repetitive element (RS2) and a 4.6-kb central core region. A 2.7-kb CTX-related element (RS1) is adjacent to the CTXΦ. The open reading frames are shown as arrows, and gray triangles represent the *attRSI* sites (adapted from Boyd and Waldor, 1999).

The CTXΦ genome also encodes the functions necessary for a site-specific integration system, and can integrate into the *V. cholerae* chromosome at a unique 17-bp attachment site, *attRS* (Pearson *et al.*, 1993). CTXΦ can undergo excision from the chromosome and replicate as a plasmid yielding single-stranded phage DNA (Waldor and Mekalanos, 1996). Recently, it was shown that CTXΦ integration is dependent on two chromosomally-encoded tyrosine recombinases, XerC and XerD, since deletion of either *xerC* or *xerD* in *V. cholerae* abolished CTXΦ integration (Huber and Waldor, 2002). *In vitro* studies have indicated that a more complex XerC/D recombinase binding site is required for CTXΦ integration, and integration probably requires additional host-encoded factors (McLeod and Waldor, 2004).

Several filamentous bacteriophages use pili as receptors for infection of host bacterial cells. In *V. cholerae*, a type IV toxin co-regulated pilus (termed TCP) necessary for bacterial colonization of the human intestines has also been found to be the functional receptor for the CTXΦ (Waldor and Mekalanos, 1996). The TCP-encoding genes are clustered on the vibrio pathogenicity island (VPI) (Karaolis *et al.*, 1998) and expression of TCP is optimal under conditions found in the intestine, suggesting that the CTXΦ may have evolved to be a highly efficient transmissible agent within the gastrointestinal environment.

## Enterotoxin operon structure

In contrast to the location of the *ctx* genes on a lysogenic phage, the genes specifying the production of Etx are found on large naturally occurring plasmids, called *ENT,* present in enterotoxinogenic strains of *E. coli* (Gyles *et al.*, 1977). These plasmids have been shown to be transmissible by conjugation, and often known to possess additional genes for drug resistance and colonization antigens (Smith and Linggood, 1971; Gyles *et al.*, 1977). Since the *E. coli* Etx genes were plasmid borne, Falkow and co-workers were able to use the emerging techniques of genetic engineering in the mid-1970s to isolate and clone the Etx genes from an ENT plasmid derived from an ETEC strain of porcine origin (So *et al.*, 1978).

The genes encoding the A- and B-subunits of Etx have been variously designated by early investigators as either *eltA/eltB* or *toxA/toxB*, with the use of subscripts H or P in some cases to denote the origin of the gene as arising from a human or porcine strain of *E. coli*. Because of these disparities in nomenclature, the authors have chosen to use a general mnemonic, *etxA* and *etxB*, for the genes encoding the A- and B-subunits of the *E. coli* heat-labile enterotoxin, irrespective of the source of the ETEC infection.

The *etx* genes from a porcine isolate (P307) and two human isolates (H10407 and H74-114) have been sequenced (Dallas and Falkow, 1980; Spicer and Noble, 1982; Yamamoto and Yokota, 1982; Yamamoto and Yokota, 1983; Yamamoto *et al.*, 1984; Yamamoto *et al.*, 1987; Leong *et al.*, 1985; Webb and Hirst, unpublished). This revealed that *etxA* and *etxB* genes overlapped one another by four nucleotides, and upon sequencing the *ctxA* and *ctxB* genes, an identical 4-bp overlap was also found (Mekalanos, 1983; Lockman and Kaper, 1983). A consensus promoter sequence for RNA polymerase binding is located upstream of *etxA*, whereas no such sequence is found in the nucleotides proximal to *etxB* (Dallas and Falkow, 1979; Dallas *et al.*, 1979). Upstream of the single promoter site proximal to the *ctxA* gene is a series of three to eight tandemly repeated copies of a TTTTGAT motif, involved in ToxR-mediated activation of Ctx expression (see below) (Mekalanos *et al.*, 1983; Miller and Mekalanos, 1984; Miller *et al.*, 1987).

## Regulation of toxin expression

Several genes required for the virulence of *V. cholerae*, including Ctx gene expression, are under the control of the ToxR protein. This is a 32.5-kDa integral cytoplasmic membrane binding protein that influences the transcription of the *ctx* operon in response to changes in several environmental factors, including aeration, osmolarity, pH, temperature, and the availability of several amino acids (DiRita, 1992; Miller and Mekalanos, 1988; Mekalanos, 1992). ToxR is essential for virulence, and increases the transcription of the *ctxAB* promoter by binding to the TTTTGAT repeat motif found upstream of the -35 region (Miller *et al.*, 1987).

ToxR function depends on its interaction with an integral membrane protein ToxS, encoded downstream of *toxR* (Miller *et al.*, 1989). It is thought that ToxS may stabilize ToxR as a homodimer in the membrane, facilitating attainment of an activated state (Miller *et al.*, 1989; DiRita and Mekalanos, 1991). In a *toxR* mutant strain of *V. cholerae*, no Ctx expression occurs; however, constitutive expression of ToxT, an AraC-like transcriptional activator, overcomes this defect, suggesting that ToxT as well as ToxR may modulate the activity of the *ctx* promoter (DiRita, 1992; DiRita *et al.*, 1996). *V. cholerae* strains with a *toxT* mutation were avirulent and unable to express Ctx or TCP (Champion *et al.*, 1997), signifying that *toxT* activation is essential for virulence.

A second two-component regulatory complex comprising of TcpPH is also involved in mediating activation of *toxT* and works in a similar manner to the ToxRS system. TcpP is essential for TCP production, and activity of TcpP is enhanced by TcpH (Figure 15.4). It has been suggested that both ToxRS and TcpPH are

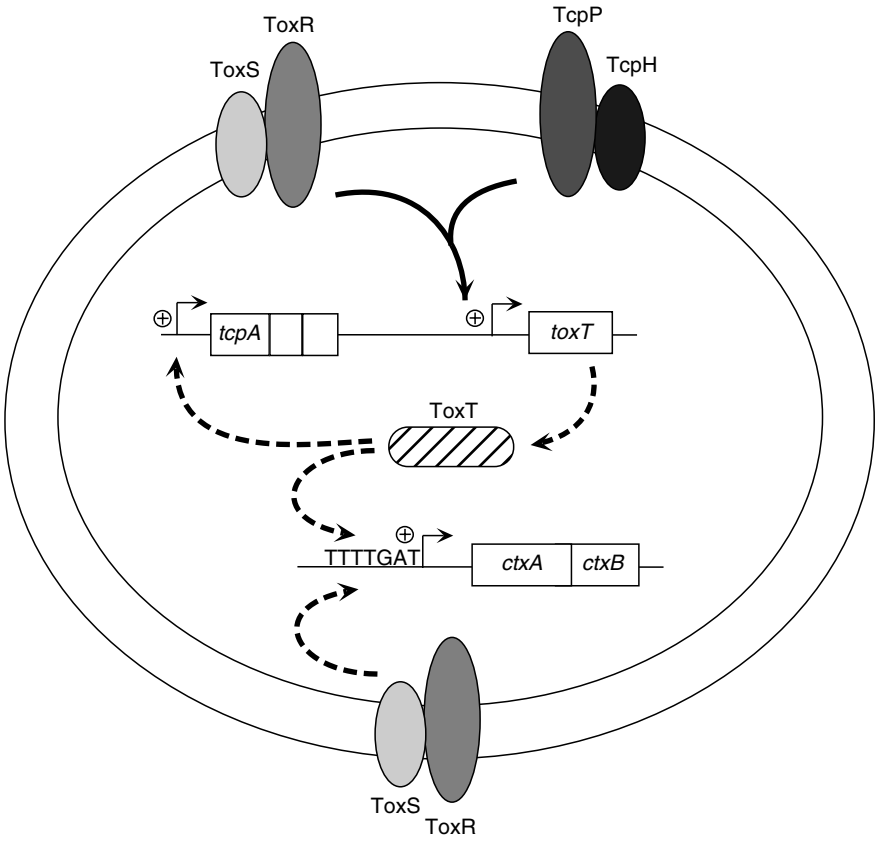



**FIGURE 15.4** Model for activation of *ctxAB* transcription. ToxR, ToxS, TcpP, and TcpH are inner membrane proteins that activate *toxT* transcription from the toxT promoter. ToxT protein then activates transcription of the *tcpA* promoter, producing more ToxT. ToxT also activates other virulence genes, such as *ctxA* and *ctxB*. ToxR can also directly activate the *ctxAB* promoter by interaction with an upstream septamer sequence $(TTTTGAT)_n$ (adapted from Yu and DiRita, 1999).

required for optimal expression of ToxT in *V. cholerae* (Hase and Mekalanos, 1998); however, the exact mechanism that TcpP and ToxR utilize for full activation of *toxT* is poorly understood.

Recently, two additional activators, AphA and AphB, were identified as regulators of the ToxR virulence cascade (Skorupski and Taylor, 1999; Kovacikova and Skorupski, 1999) and thought to interact directly with each other resulting in *tcpPH* activation (Kovacikova *et al.*, 2004). Another two-component bacterial signal transduction system, VieSAB, was identified as a positive regulator of *ctxAB* (Lee *et al.*, 2001) and may modulate *ctxAB* expression through influencing transcription of *toxT* (Tischler *et al.*, 2002). In addition, the histone-like nucleoid-associated protein (termed H-NS) has been shown to repress several genes within the ToxR virulence regulon, including the *toxT, tcpA*, and *ctx* promoters (Nye *et al.*, 2000; Nye and Taylor, 2003). Such ongoing extensive studies of this complex regulatory system will provide further insight into the nature of toxin expression and the initiation of the virulence cascade that leads to such a potent and devastating infection.

The expression of *E. coli* heat-labile enterotoxin is influenced by growth temperatures, with an approximated fourfold elevation in Etx production at 37˚C compared to 30˚C (Hirst and Uhlin, unpublished observations). While this could be due to an influence on any one of the multiple steps in toxin biogenesis, it is thought that this is mediated at the level of *etx* mRNA transcription as a result of changes in the local topology of DNA caused by H-NS.

## Translational control of subunit stoichiometry

Translation of the polycistronic *ctx* or *etx* mRNAs that arise from transcriptional initiation upstream of the *ctxA* or *etxA* cistrons is the start of events that lead to the synthesis of the A- and B-subunits.

Initially, the subunits are produced as precursor polypeptides (preA- and preB-) that then undergo export across the cytoplasmic membrane (see below). Since the subunit structure of the assembled toxins is 1A:5B-subunits, this implies that the synthesis of the A- and B-subunits may be stoichiometrically controlled in some way. A careful examination of the relative levels of A- and B-subunit synthesis in *E. coli* expressing either porcine or human Etx indicated that one to two A-subunits are synthesized per five B-subunits (Hofstra and Witholt, 1984; Hirst and Hardy, unpublished observations). The

molecular mechanisms responsible for this have, as yet, not been comprehensively investigated.

The fact that both subunits are expressed from a polycistronic mRNA means that the relative levels of A- and B-subunit synthesis must be governed by events that occur after transcriptional initiation. The most likely explanation is that differences in the efficiency of the Shine-Dalgarno (SD) sequences just upstream of the start codons of the A- and B-cistrons influence translational initiation, although other explanations, such as the presence of local stable secondary mRNA structures interfering with or promoting ribosomal recognition of the SD sequence or ribosome movement along the mRNA, cannot be excluded. An analysis of the SD sequences upstream of the initiation codons of the A- and B-subunits of Ctx and Etx revealed clear differences in the number of nucleotides of the A- and B-cistrons that share complementarity with the SD consensus sequence (5′-AGGAGG-3′), which would be consistent with the higher level of B subunit synthesis being due to the presence of a more efficient ribosome-binding site adjacent to the B-subunit cistron.

## Toxin export across cytoplasmic membrane

Protein export across the cytoplasmic membranes of bacteria, especially in *E. coli*, has been extensively investigated over the past 30 years. Protein translocation is dependent on both distinct structural properties of exported proteins, such as the presence of the amino-terminal signal peptides, and a highly efficient translocation machinery that not only delivers exported proteins to the membrane but also achieves their translocation across it.

Our current perception of how the A- and B-subunits of Etx and Ctx cross the cytoplasmic membranes of *E. coli* and *V. cholerae* is based primarily on an understanding of protein export in general, in laboratory strains of *E. coli* K–12. The precise molecular mechanism by which polypeptides translocate across the cytoplasmic membrane remains to be elucidated, but based on current concepts, it seems most likely the polypeptide is threaded like a "string of beads" through a Sec translocation channel in an energy-dependent process requiring both ATP and proton motive force (pmf).

The signal peptides present at the amino-termini of the precursor A- and B-subunits of Ctx/Etx are 18 and 21 amino acids long, respectively (Figure 15.1). A comparison of the signal peptides encoded by the *ctx* and *etx* genes and between *etx* genes of human and porcine origin shows that the signal sequences are less well conserved than the mature sequences (Figure 15.1) (Mekalanos *et al.*, 1983; Yamamoto *et al.*, 1987; Dams *et al.*, 1991). This is not too unexpected, since there is considerable sequence diversity among signal peptides in general, even though they perform a conserved function (Randall and Hardy, 1989).

To date, eight Sec proteins are known to be involved in the targeting and translocation of precursor proteins across bacterial cytoplasmic membranes (Driessen *et al.*, 1998). These are SecA (a translocation ATPase), SecB (a molecular chaperone), SecE, G, and Y (which form a trimeric membrane), SecD, F, and the largely uncharacterized YajC (also located in the cytoplamic membrane, although SecD and SecF have large periplasmic loops) and LepB (leader peptidase II—responsible for cleaving off the signal peptide from the precursor molecule). Similar proteins have been identified in other Gram-negative and Gram-positive bacterial species (Pugsley, 1993; Simonen and Palva, 1993), although as yet, the respective homologues in the *Vibrionaceae*, including *V. cholerae*, have not been characterized.

Protein export is an energy-dependent process, requiring both ATP and pmf (Driessen *et al.*, 1998; Geller, 1991). Early studies on the biogenesis of Etx in *E. coli* showed that in the presence of uncouplers of pmf, unprocessed precursor B-subunits accumulated (Palva *et al.*, 1981). The ATP requirement for export across the cytoplasmic membrane is presumed to be due solely to the activity of SecA. It is now accepted that ATP and pmf function at different stages of the translocation process, with ATP playing an essential role in the early events of precursor insertion and translocation, while pmf completes the process. However, the exact function of pmf in translocation remains to be precisely determined, especially in light of the findings that different precursors appear to require different levels of pmf for efficient export (Daniels *et al.*, 1981; Driessen and Wickner, 1991; Driessen, 1992).

The last step in the export of the toxin subunits across the cytoplasmic membrane of both *E. coli* and *V. cholerae* involves their release from the translocation channel into the aqueous environment of the periplasm. This step coincides with the important events of subunit folding and the acquisition of tertiary structure. Interactions between secondary structural elements are likely to begin as soon as the amino-terminal portion of the polypeptide emerges from the translocation channel, and will lead to the formation of loosely packed tertiary domains. If the polypeptide is exported co-translationally, it is conceivable that a significant degree of folding may have occurred on the periplasmic face of the cytoplasmic membrane, before the carboxy-terminal portion of

the polypeptide has been synthesized. The release of polypeptides from the membrane may, in fact, be dependent on the acquisition of a soluble tertiary-like structure, since parameters, such as low temperature (which slows down folding) or the use of truncated proteins that do not fold correctly, can result in the failure to release the polypeptide from the membrane (Ito and Beckwith, 1981; Koshland and Botstein, 1982; Hengge and Boos, 1985; Minsky *et al.*, 1986; Fitts *et al.*, 1987; Sandkvist *et al.*, 1987; Sandkvist *et al.*, 1990).

Pulse-chase experiments, using [35S]-methionine, have been used to follow the kinetics of toxin subunit export and release into the periplasm of *E. coli* and *V. cholerae* (Hirst *et al.*, 1983; Hofstra and Witholt *et al.*, 1988). This revealed that, in common with other proteins, export of the B-subunits of porcine and human Etx was very fast: release was complete within 10 s. In contract, Hofstra and Witholt (1984) showed that release of mature A-subunits occurred much more slowly, with up to a third remaining membrane-associated 3 min after the initiation of the pulse-chase. Given that these studies used a recombinant plasmid, EWD299, which produced porcine EtxA- and EtxB-subunits in a molar ratio of approximately 2:5, it is conceivable that if A-subunits are expelled from the translocase but fail to associate with B-subunits, they may then irreversibly re-associate with the membrane. This certainly occurs for mutant B-subunits with minor deletions or substitutions at their carboxy-termini or mutations in the Cys-residues, which prevent correct assembly (Sandkvist *et al.*, 1990; Hardy and Hedges, 1996).

## Toxin folding and assembly

The folding and assembly of the A- and B-subunits to form a final toxin structure clearly represent key molecular processes in toxin biogenesis. While the events of folding and assembly can be thought of as distinct processes, it is perhaps more appropriate to view them as a pathway of events involving both intra- and inter-molecular protein interactions.

During assembly, the intramolecular interactions that give rise to tertiary structure in the individual toxin subunits will create interfaces that will allow specific intermolecular interactions that ultimately lead to stable quaternary complexes. The nature of the interactions between B-subunits, and between the A and B-subunits, affords the possibility that these associations may affect late folding events. It is also important to consider that the folded B-subunit monomer contains two surfaces that extensively pack against adjacent B-subunits in the assembled toxin (Figure 15.2) (Sixma *et al.*, 1993). Since these surfaces are particularly hydrophobic, it is possible that the folded monomer may require "shielding" by chaperone-like proteins to prevent them from aggregating or non-specifically associating with the membrane.

The A- and B-subunit polypeptides of Ctx and Etx undoubtedly begin to fold as soon as they emerge from the translocase channel, although it is not yet clear to what extent they achieve a folded tertiary structure before being fully released into the periplasm. Recently, it was demonstrated that the formation of the disulfide bond in the B-subunit was critically dependent on a periplasmic enzyme, DsbA (Peek and Taylor, 1992; Yu *et al.*, 1992). The periplasmic location of this enzyme suggests that this late step in folding of the B-subunit occurs in the periplasm. Furthermore, it has been shown by pulse-chase experiments that the release of toxin subunits into the periplasm precedes the appearance of assembled oligomers (Hirst *et al.*, 1983; Hofstra and Witholt, 1984; Hirst and Holmgren, 1987b). As a result of these observations, it has been proposed that toxin assembly occurs largely within the periplasmic compartment of the bacterial envelope, although the possibility remains that certain subunit-subunit interactions may start at the membrane (Hirst and Leece, 1991).

In common with many other exported and secreted polypeptides, the A- and B-subunits of Etx and Ctx possess cysteine residues that must oxidize to form specific intrachain disulfide bonds during folding. Each of the toxin subunits possesses a single disulfide bond, between Cys 187 and Cys 199 in the A-subunit and between Cys 9 and Cys 86 in each B-subunit (Sixma *et al.*, 1993). The importance of the formation of disulfide binds in toxin biogenesis was first demonstrated by the use of the sulfhydryl reducing reagent dithiothreitol (DTT), which immediately stopped the assembly of new EtxB-pentamers in an EtxB-producing strain of *E. coli*, although it had no effect on the pentamers that had already assembled. The subsequent demonstration that substitution of Cys 9 or Cys 86 by Ser in the B-subunit of Ctx and Etx (Jobling and Holmes, 1991; Hardy and Hedges, 1996) abolished B-subunit assembly into stable oligomers confirmed that disulfide bond formation in the B-subunit is an essential step in toxin biogenesis.

It is now known that the formation of disulfide binds in the enterotoxin molecule is catalyzed by a periplasmic thiol-disulfide oxidoreductase (Yu *et al.*, 1992; Bardwell, 1994; Missiakas and Raina, 1997). Genes encoding analogous enzymes responsible for disulfide bond formation (*dsbA*) have been identified in *E. coli*, *V. cholerae*, and *Haemophilus influenzae* (where the mnemonic *ppfA*, *tcpG*, and *por* have also been used) (Bardwell *et al.*, 1991;

Kamitani *et al.*, 1992; Peek and Taylor, 1992; Tomb, 1992; Yu *et al.*, 1992).

The *cis-trans* isomerization of peptidylprolyl bonds is recognized to be a slow, rate-limiting step in the refolding of proteins *in vitro* (Jaenicke, 1987; Jaenicke, 1991; Lang *et al.*, 1987). Peptidylprolyl *cis-trans* isomerases are located in the cytoplasm and the periplasm of bacteria and are thought to accelerate *cis-trans* prolyl isomerization *in vivo* (Liu and Walsh, 1990; Hayano *et al.*, 1991; Missiakas and Raina, 1997; Dartigalongue and Raina, 1998). Equilibrium is established between the *cis* and the *trans* states of the monomeric EtxB under denaturing conditions, resulting in the generation of assembly-competent (*cis*) and assembly-incompetent (*trans*) forms (Cheesman *et al.*, 2004). Each B-subunit contains a single *cis*-proline residue at position 93 (P93) and a single tryptophan residue at position 88 (W88) revealed by X-ray crystallography. Recently, mutagenesis of the P93 to a glycine or an alanine residue revealed minor differences in their biophysical and biochemical properties, but major changes in the kinetics of pentamer disassembly and reassembly, relative to wild-type protein. These substitutions resulted in a *cis* peptide bond between amino acids 92 and 93, suggesting that the adoption of the *cis* conformation is a prerequisite for efficient production and secretion of these recombinant proteins (Cheesman *et al.*, 2004).

The precise pathway of subunit interactions and the identity of assembly intermediates that lead to the formation of an $AB_5$ holotoxin complex are not yet known. The B-subunits of Ctx and Etx readily assemble in the periplasm (in the absence of concomitant A-subunit synthesis) to form stable pentamers (Hirst *et al.*, 1984). This should not be taken to imply that assembly involved the formation of the B-pentamer before association with the A-subunit, since there is compelling evidence that the A-subunit interacts with B-subunits before pentamerization is complete.

Although the exact pathway of B-subunit pentamerization is not known, it has been speculated that a preferred pathway might involve the initial dimerization of two B-subunit monomers, followed by incorporation of a further monomer, and culmination in the association of a further dimer to form a stable pentameric structure (Hirst *et al.*, 1995). A kinetic simulation of the pathway of CtxB pentamerization has recently been modeled based on monitoring changes in Trp-fluorescence during assembly *in vitro* (Lesieur *et al.*, 2002).

The association of the A- and B-subunits to form an $AB_5$ holotoxin adds another level of complexity to the assembly process. The crystal structure of the holotoxin gives a picture of the final outcome of these interactions. The major contacts between the A- and B-subunits are clustered towards the C-terminal portion of the A2-polypeptide, which is inserted into the central pore of the B-pentamer where there are five salt-bridge interactions (Sixma *et al.*, 1993) (Figure 15.2). Within the central pore, there are surprisingly few specific contacts: just two regions of hydrophobic contact at each end of the pore and three salt bridges between three adjacently positioned B-subunits (Sixma *et al.*, 1993).

*In vivo* studies on Etx assembly in *E. coli* have revealed that the rate at which B-subunits attain a pentameric structure in the periplasm is increased by roughly fourfold when the A-subunit is co-expressed (Hardy *et al.*, 1988). This has been interpreted to mean that the A-subunit stabilizes an intermediate in B-subunit assembly. Although several pathways for A-/B-subunit assembly can be envisaged, it seems plausible to hypothesize that assembly of the A- and B-subunits proceeds via the formation of an $AB_3$ intermediate. Indeed, it may be the stabilization of the B-trimer by the A-subunit that leads to the enhancement in the rate of B-subunit pentamerization observed by Hardy *et al.* (1988). If the generation of the $AB_3$ intermediate is part of the normal pathway of toxin assembly, then its subsequent association with a preformed B-dimer would form the holotoxin complex.

## Toxin secretion across OM

*V. cholerae* has a distinct secretory apparatus capable of translocating fully folded and assembled proteins across its outer membrane, including Ctx. Studies on the export and secretion of the A- and B-subunits of Ctx from strains engineered to express one or the other of the subunit toxins led to the conclusion that the assembled B-pentamer contains all of the necessary structural determinants to permit secretion across the *V. cholerae* outer membrane (Hirst *et al.*, 1984; Hirst and Holmgren, 1987a). Subsequently, Sandkvist and colleagues showed that Ctx is secreted via the extracellular protein secretion (Eps) system, a type II secretion pathway (Sandkvist, 2001b), previously referred to as the main terminal branch of the general secretion pathway (GPS) (Pugsley *et al.*, 1997). The extracellular localization of proteins transiting two membranes occurs via a two-step mechanism; first, the Sec machinery translocates unfolded proteins through the inner membrane into the periplasm, where folding and posttranslational modification take place, and second, the subsequent translocation of the folded protein through the outer membrane is mediated by Eps proteins (Sandkvist *et al.*, 1999; Sandkvist *et al.*, 2000; Russel, 1998) (Figure 15.5).

The genetic organization of type II secretion systems is highly conserved among Gram-negative bacterial

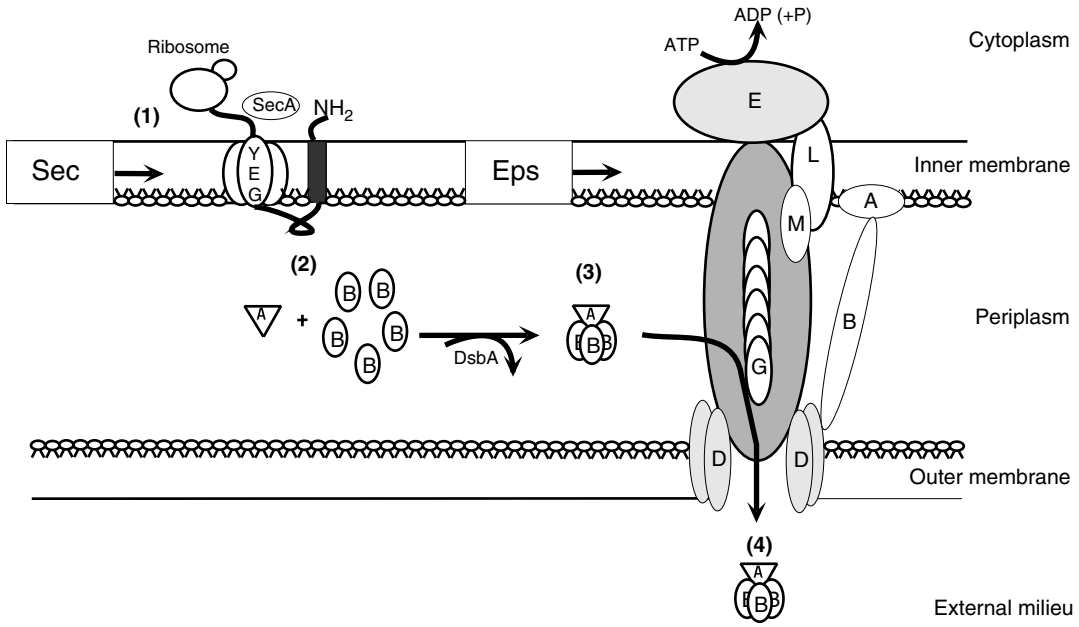



**FIGURE 15.5** Schematic model of the pathway of toxin biogenesis in *E. coli* (steps 1–3 only) and in *V. cholerae* (steps 1–4). Step (1), precursor A- and B-subunits are exported across the cytoplasmic membrane via a Sec-dependent {58}pathway involving SecA, SecE, SecY, and EcG (SecD, SecF, YajC not shown), followed by their cleavage by LepB (not shown) to yield mature A- and B-subunits that are released into the periplasm. Step (2), folding of the mature subunits is mediated both by intramolecular interactions within the polypeptide chain and by intermolecular interactions with factors that may facilitate or catalyze folding events, such as DsbA and peptidyl prolyl cis-trans isomerase. Step (3), holotoxin assembly occurs to give rise to an $AB_5$ structure. Step (4) the $AB_5$ complex engages with the type II secretion apparatus via specific recognition of $B_5$, and is secreted across the outer membrane via the Eps-secretion machinery. The function of the various Eps proteins is still to be resolved, but it is speculated that EpsG-K may form a retractable pilus that participates like a piston in pumping the toxin through the secretion pore, comprised of EpsD. It is likely that the secretion pore is gated to prevent the non-specific loss of resident proteins or the influx of low molecular mass solutes. Energy in the form of ATP and pmf is required for holotoxin translocation across the outer membrane. (adapted from Sandkvist, 2001a)

species including *V. cholerae*. The genes are organized in a single operon (see Sandkvist, 2001a for a comprehensive review of known type II secretion pathway genes). To date, 15 *eps* genes have been identified in *V. cholerae* essential for type II secretion, with the gene products designated by letters A through O. Since the function and interaction of all the proteins in the type II secretion system have been reviewed (Sandkvist, 2001a; Sandkvist, 2001b), it will not be discussed in great detail. These EPS proteins are thought to form a large multiprotein complex that spans across the periplasmic compartment and enables the translocation of proteins through the outer membrane. EpsL and EpsM are thought to be responsible for opening and closing the secretion pore (Sandkvist *et al.*, 1999), while EpsD present in the outer membrane mediates both Ctx and CTXΦ secretion (Davis *et al.*, 2000). Subsets of these *eps* genes (*epsD*, *epsE*, *epsF*, and *epsO*) are homologous to the genes required for assembly and export of type IV pili, and other *eps* genes (*epsG epsH*, *epsI*, *epsJ*, and *epsK*) encode proteins similar to type IV prepilin subunits. It has been suggested that these *eps* gene products represent a subset of the type II secretion system with the capability of forming a pilus-like structure (Sandkvist, 2001a). The structure of some EPS proteins from *V. cholerae* has been elucidated, including EpsE (Robien *et al.*, 2003), EpsM (Abendroth *et al.*, 2004b), and EpsL (Abendroth *et al.*, 2004a), and it has provided insight into the functional aspect of these proteins. However, much remains to be done to fully comprehend the sophisticated mechanism by which these toxins are secreted.

Although the exact mechanism by which Ctx is secreted still remains unknown, different models for pilus-mediated secretion have been proposed. Recent studies have shown that type IV pili can forcefully retract (Merz *et al.*, 2000) with subunits assembling into filaments prior to translocation (Wolfgang *et al.*, 2000). This suggests that type II secretion pilus may act as a piston, pushing secreted proteins through a gated pore (Sandkvist, 2001a). A source of energy in the form of proton-motive force or ATP hydrolysis is essential, and may be required for opening of the secretion pore or assembly of the secretion apparatus (Figure 15.5).

Studies on *Aeromonas hydrophilia* have revealed that ATP hydrolysis by ExeA is necessary for aerolysin secretion (Pugsley *et al.*, 1997) and two homologues of *exeA* have been found in *V. cholerae* (Hillary and Hirst, unpublished observations). It is speculated that EpsE, which is an ATP binding protein, plays an important role in linking ATP hydrolysis to the process of toxin secretion (Sandkvist *et al.*, 1995; Sandkvist, 2001a). However, the role of energy and how this is coupled to the secretory event still needs to be explored. Once released from the microorganism, either by secretion or by non-specific lysis, the toxin is free to interact with the epithelium of the gut and initiates the events that lead to toxin-mediated diarrhea.

## ACTION OF CHOLERA TOXIN AND RELATED ENTEROTOXINS IN MEDIATING DIARRHEA

Ctx and Etx exert their diarrheagenic action by subverting the normal physiological processes of gut epithelial cells. To do so, the toxins must bind to and enter the cells of the gut epithelium.

### Cell surface receptor binding

The B-subunit moiety of Ctx and Etx are lectins that enable them to bind to cell surface receptors (refer to de Haan and Hirst, 2004 for a recent review). The principal receptor for Ctx is GM1-ganglioside (Gal(β1-3)GalNAc (β1-4)[NeuAc(α2-3)]Gal (β1-40)Glc(β1-1)ceramide), a glycosphingolipid found ubiquitously on the surface of eukaryotic cells (Van Heyningen *et al.*, 1971; Cuatrecasas, 1973; Holmgren, 1973). The X-ray structure of CtxB bound to the pentasaccharide moiety of GM1 revealed that each B-subunit has a GM1 binding pocket (Merritt *et al.*, 1994; Merritt *et al.*, 1998). Upon receptor binding, a flexible loop comprising amino acids 51–58 becomes more ordered, stabilizing the toxin-GM1 complex (Merritt *et al.*, 1994).

High-affinity interaction between CtxB and the pentasaccharide moiety of GM1 is due to hydrogen bond interactions with the terminal Gal and sialic acid (NeuAc) residues, and to a more limited extent with GalNAc of GM1. This results in an exceptionally high affinity with reported dissociation constants of ($K_{D'S}$) of 7.3 x $10^{-10}$M for CtxB and 5.7 x $10^{-10}$M for EtxB (Kuziemko *et al.*, 1996). In addition to GM1, CtxB is reported to bind with lower affinity to other receptors, such as GD1b, GQ1b, GD1a, GT1b, and GM2 (Fukuta *et al.*, 1988; Angstrom *et al.*, 1994; Kuziemko *et al.*, 1996; Lauer *et al.*, 2002). EtxB displays a more promiscuous receptor-binding activity and in addition to binding to the gangliosides above, also interacts with asialo-GM1, non-ganglioside glycolipid receptors, lactosylceramide, and certain galactoproteins (Orlandi *et al.*, 1994; Teneberg *et al.*, 1994; Karlsson *et al.*, 1996; Backstrom *et al.*, 1997). The significance of these differential binding specificities on the toxicity properties of Ctx and Etx has yet to be fully evaluated.

Studies on the enterotoxicity of a GM1-binding mutant of Etx, Etx(G33D), which contains a Gly33 to Asp substitution in the B-subunit, confirmed that GM1 binding is essential for toxin action (Tsuji *et al.*, 1985; Jobling and Holmes, 1991; Guidry *et al.*, 1997; Nashar *et al.*, 1998). However, GM1 binding alone may not be sufficient to ensure full expression of toxicity, since a mutational analysis of the 51–58 loop revealed that substitution of His57 to Ala resulted in a mutant B-subunit that retained its GM1-binding properties, but its capacity to induce electrogenic chloride secretion in model epithelia was ablated (Aman *et al.*, 2001; Rodighiero *et al.*, 2002; Fraser *et al.*, 2003).

Recently, Etx bound to lipopolysaccharide on the bacterial surface was shown to be able to bind to GM1, a finding that may suggest the toxin is able to mediate the delivery of associated somatic antigens or other virulence determinants at the same time as the toxin enters enterocytes (Horstman and Kuehn, 2002; Horstman *et al.*, 2004). Moreover, an immunofluorescence analysis of the interaction of ETEC vesicles with intestinal epithelial cells has suggested that toxin-containing vesicles may be the primary means of entry of active Etx into host cells (Kesty *et al.*, 2004).

### Uptake and trafficking of toxin

The use of polarized human colonic epithelial T84 cells (Lencer *et al.*, 1992) has greatly facilitated studies of Ctx and Etx toxin action, since toxicity can be readily monitored as the induction of electrogenic $Cl^-$ secretion. GM1 ganglioside is clustered in detergent insoluble glycosphingolipid-rich membrane microdomains (DIGs), which play a significant role in receptor-mediated endocytosis and signal transduction. The binding of Ctx to GM1 ganglioside within DIGs is a critical step in the targeting of the toxin into intracellular compartments (Wolf *et al.*, 1998; Wolf *et al.*, 2002; Orlandi and Fishman, 1998). An early study showed that cholesterol derivatives of GM1 were more effective receptors for Ctx action than native GM1 or phospholipid derivatives of GM1 (Pacuszka and Fishman, 1991) and treatment of T84-cells with cholesterol-depleting drugs such as filipin or β-cyclodextrin reversibly inhibited Ctx-induced $Cl^-$ secretion (Orlandi and Fishman, 1998; Wolf *et al.*, 1998;

Wolf *et al.*, 2002). Several other studies have also suggested that cholesterol may be involved in the recruitment of lipid raft constituents that interact with and facilitate the internalization of Ctx-GM1 complexes in a clathrin-independent manner (Torgersen *et al.*, 2001; Nichols, 2002). The disruption of the actin cytoskeleton by drugs such as Cyto-D resulted in intact lipid rafts but loss of cytoskeleton-dependent lipid raft functions (Badizadegan *et al.*, 2004), suggesting that the actin cytoskeleton also plays a role in trafficking of Ctx.

Once Ctx is internalized in endocytic vesicles (AE), the toxin trafficks to the trans-Golgi network (TGN) and then enters the endoplasmic reticulum (ER) (Figure 15.6) (Sandvig *et al.*, 1994; Nichols and Lippincott-Schwartz, 2001; Lencer and Tsai, 2003). The presence of a KDEL motif at the C-terminus of CtxA (RDEL in EtxA), although not essential for toxicity, increases the efficiency of retrograde trafficking into the ER (Lencer *et al.*, 1995). A well-characterized pathway of retrograde transport is via COPI-coated vesicles (Cosson and Letourneur, 1997). This large cytosolic protein complex forms a coat around vesicles budding from the Golgi, and utilizes a GTP-switch mechanism for coating and uncoating (Barlowe, 2000). Conversely, anterograde transport into the Golgi from the ER, termed *COPII*, is mediated by a distinct coated vesicle and also employs a similar coating mechanism (Barlowe, 2000). The retrograde cargo of COPI vesicles includes ER-resident proteins that carry a C-terminal KDEL-motif and interact with the abundantly expressed KDEL receptor (or Erd2p) (Munro and Pelham, 1987; Pelham, 1988; Tang *et al.*, 1993; Griffiths *et al.*, 1994; Orci *et al.*, 1994; Majoul *et al.*, 1998). The active domains of a number of bacterial toxins, such as the A-subunit of Ctx, contain a C-terminal KDEL sequence, and it is likely that retrograde transport of these toxins through the ER involve COPI-coated vesicles. Studies have shown that the movement of Ctx A-subunits into the ER was blocked by microinjection of antibodies specific for subunits of the COPI coat (Majoul *et al.*, 1998). Moreover, trafficking of Ctx depends on the p24 family proteins that are sites for COPI coatomer binding (Stamnes *et al.*, 1995), further confirming the involvement of these vesicles in the trafficking of Ctx.

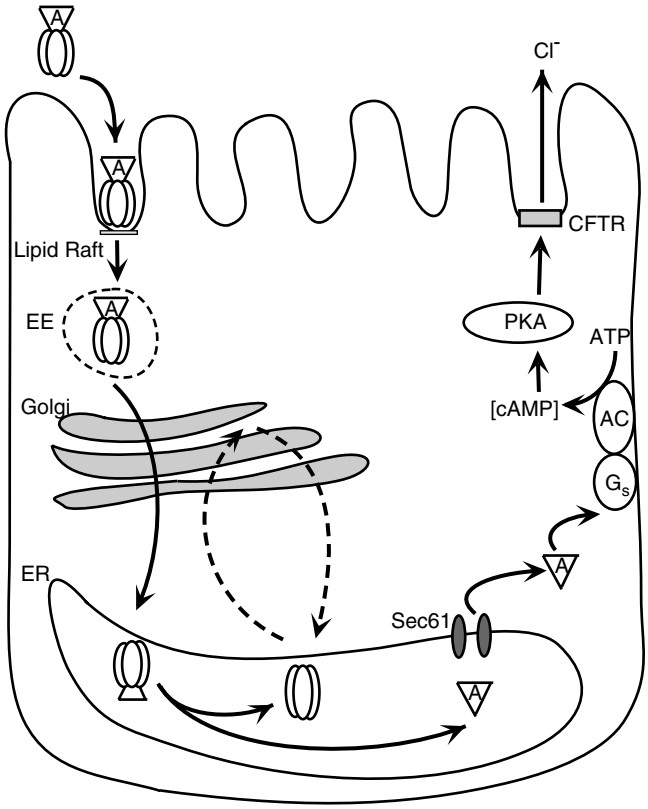



**FIGURE 15.6** Schematic model of toxin uptake and action in polarized epithelia. The cholera toxin (CT) binds to GM1 and enters via a lipid raft. After endocytosis, the CT may move from the early endosome (EE) directly to the Golgi and then to the endoplasmic reticulum (ER). In the ER, the A1 fragment unfolds and dissociates from the B-pentamer, and is then translocated to the cytosol through the protein conducting channel. As the A1 fragment moves into the cytosol, it avoids ubiquitination, and rapidly folds into and exzymatically active state enabling it to ADP ribosylate the GTP binding protein, Gs. Activated Gs stimulates the adenylate cyclase complex (AC), leading to a concomitant rise in cyclic AMP (cAMP). This in turn causes activation of protein kinase A (PKA), which phosphorylates the major chloride channel, (CFTR), resulting in a CT secretory response, the primary event responsible for causing diarrhea.

Although these COPI vesicles are thought to be involved in Ctx trafficking, there has been some evidence suggesting the contrary. It was found that CtxB and EtxB alone were able to traffic to the various Golgi cisternae and do not appear to require the KDEL sequence (Parton, 1994; Sandvig *et al.*, 1994; Sandvig *et al.*, 1996). Furthermore, a mutation or replacement of KDEL in Ctx (or RDEL in Etx) resulted in a delay, but not complete inhibition of toxin-induced $Cl^-$ secretion and trafficking to the basolateral membrane (Lencer *et al.*, 1995; Cieplak *et al.*, 1995) and recently, treatment of Vero cells with a drug that interacts with COPI coatomer inhibited binding to Golgi membranes and subsequently failed to inhibit Ctx trafficking to the ER (Chen *et al.*, 2002). These findings suggest that Ctx may be transported in COPI-coated vesicles without necessarily binding to the KDEL receptor. Alternative trafficking routes for Ctx may possibly exist, as studies on Shiga toxin (which does not have a KDEL sequence) were found to utilize a COPI-independent retrograde pathway controlled by a small G protein, Rab6 (Girod *et al.*, 1999; White *et al.*, 1999). Furthermore, Ctx-induced toxicity was unaffected despite the inhibition of multiple endocytic pathways for Ctx trafficking into

the ER, suggesting that an additional retrograde trafficking pathway may exist (Massol *et al.*, 2004).

It has been speculated that the A1 fragment dissociates from the A2-B pentamer complex in the ER through the action of protein disulfide isomerase (PDI), which has both a thiol-disulphide oxidoreductase activity and a capacity to act as an unfoldase (Tsai *et al.*, 2001). PDI catalyzes reduction of the disulphide bridge between Cys-187 in the A1 fragment and Cys-199 in the A2 fragment, and forms a transient intermediate adduct with the A1 fragment that must then be oxidized in order for the A1 fragment to be released and translocated across the ER membrane. Recently, the ER oxidase Ero1 was found to be responsible for inducing release of the A1 fragment by mediating oxidation of the carboxy-terminal disulfide bond in PDI (Tsai and Rapoport, 2002). The complex of PDI and unfolded toxin is targeted to the luminal side of the ER for translocation across the membrane, since the A1 fragment alone does not have a high affinity for the ER (Tsai and Rapoport, 2002).

The subsequent translocation of the unfolded A1 fragment into the cell cytosol is thought to occur via direct association with the Sec61p secretion channel found in the ER membrane (Hazes and Read, 1997; Schmitz *et al.*, 2000). The Sec61p protein complex is involved in the transport of newly synthesized proteins from the cytosol into the ER, as well as transport of misfolded proteins from the ER for proteasomal degradation in the cytosol (Kopito, 1997; Matlack *et al.*, 1998). It would appear that the toxin takes advantage of this protein quality control machinery of cells for handling misfolded proteins, known as *ER-associated degradation* (ERAD), to achieve its translocation to the cytosol (Werner *et al.*, 1996; Teter and Holmes, 2002; Tsai and Rapoport, 2002). However, once translocated into the cytsol, the A1 fragment does not undergo proteasomal degradation since the A1 chain has a paucity of lysines, which are sites of ubiquitination and proteasomal targeting (London and Luongo, 1989; Hazes and Read, 1997). Recent findings have revealed that the A1 fragment may undergo some ubquitin-independent degradation, presumably by other cytosolic proteases (Teter *et al.*, 2002).

Once translocated, the A1 fragment interacts with ADP-ribosylation factors (ARFs) (Moss *et al.*, 1993) that influence its catalytic activity and may serve to efficiently target the A1 fragment to the plasma membrane-associated G-proteins that are the primary target of the A1 fragment. ARFs are members of a highly conserved multigene family of small GTP binding proteins and interact with several other proteins involved with membrane trafficking (Moss and Vaughan, 1995). ARF proteins were originally identified as cofactors in Ctx-catalyzed ADP-ribosylation of G-proteins (Kahn and Gilman, 1986), but since then have been shown to be widely expressed in most mammalian tissues (Tsuchiya *et al.*, 1991). It has been found that ARFs do not interact with native Ctx; instead, they only interact after nicking and reduction of CtxA has occurred, leading to the exposure of an ARF-binding site on the A1 fragment of Ctx (Moss *et al.*, 1993). ARF6, the most divergent member of the ARF family, appears to function in the peripheral plasma membrane (D'Souza-Schorey *et al.*, 1995; Radhakrishna *et al.*, 1996) and utilizes the GTP cycle to regulate the membrane traffic pathway (Radhakrishna and Donaldson, 1997). The functional interaction between the A1 fragment and human ARF6 is essential for maximal toxicity of Ctx (Jobling and Holmes, 2000). Critical residues in the A1 fragment involved in this intricate interaction have been identified, and lead to exposure of four aromatic residues thought to create an initial ARF interaction motif (Jobling and Holmes, 2000).

Bacterial exotoxins utilize a common mechanism to ADP-ribosylate specific host proteins, and the A1 fragment of Ctx is known to irreversibly ADP-ribosylate a trimeric GTP-binding protein $G_{s\alpha}$. The formation of an A1 fragment-ARF-$G_{s\alpha}$ complex is involved in the transfer of the ADP-ribose moiety of $NAD^+$ to Arg201 in $G_{s\alpha}$ (Gill, 1975; Moss *et al.*, 1976; Van Dop *et al.*, 1984; Bourne *et al.*, 1991), causing an inversion of stereochemical configuration in the N-ribosidic bond and release of nicotinamide. This ADP-ribosylation reaction results in the inhibition of the intrinsic GTPase property of $G_{s\alpha}$ (Hepler and Gilman, 1992), thus enabling $G_{s\alpha}$ to bind GTP and activate adenylate cyclase. This leads to the continuous stimulation of the adenylate cyclase complex and a concomitant massive elevation of intracellular cAMP levels.

The high levels of cAMP cause the activation of protein kinase A (PKA), which phosphorylates the major chloride channel identified in intestinal epithelial cells, namely, the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in active chloride secretion (Field *et al.*, 1989; Burch *et al.*, 1988; Cheng *et al.*, 1991; Denning *et al.*, 1992; Picciotto *et al.*, 1992) (Figure 15.6). In addition to activation of the apically located chloride channel, the basolaterally located Na/K/2Cl co-transporter is also inhibited (McRoberts *et al.*, 1985; Halm *et al.*, 1988; Matthews *et al.*, 1992) and the $Cl^-$ secretory response is amplified by the release of prostaglandins (PGs) (Kimberg *et al.*, 1971; Speelman *et al.*, 1985; Burch *et al.*, 1988; Peterson and Ochoa, 1989; Thielman *et al.*, 1997). The inhibition of micro-tubule organization and a remodelling or dissociation of actin filaments in intestinal epithelial cells results in apical membrane recruitment of the CFTR (Shapiro *et al.*,

1991; Tousson *et al.*, 1996), and a widening of tight junctions further contributes to the loss of electrolytes (Field *et al.*, 1972; Powell, 1974). The resultant net increase in electrogenic $Cl^-$ secretion and inhibition of $Na^+$ absorption leads to osmotic movement of a large quantity of water into the intestinal lumen and results in severe secretory diarrhea. With appropriate intravenous fluid and electrolyte replacement therapy, the most severely affected cholera patients have been known to purge in excess of 80 litres of diarrhea. Most cases of *E. coli*-mediated diarrhea are less severe, and may be attributable to a whole range of parameters that influences the relative pathogencity of ETEC versus *V. cholerae*.

Interestingly, a comparison of the relative toxicity of Ctx and Etx in polarized human epithelial (T84) cells has revealed that Ctx is the more potent of the two toxins. The underlying structural basis for this difference in toxicity was determined by engineering a set of mutant and hybrid Ctx/Etx toxins (Rodighiero *et al.*, 1999). This revealed that the differential toxicity of Ctx and Etx was not due to differences in the A-subunit C terminal KDEL targeting motif (RDEL in Etx), since a KDEL to RDEL substitution had no effect on Ctx activity. Moreover, it could not be attributed to either the enzymically active A1 fragment, since hybrid toxins in which the A1 fragment of Ctx was substituted for that of Etx had exactly the same potency as that of authentic Ctx, or the B-subunit, since the replacement of the B subunit in Ctx for that of Etx caused no alteration in toxicity. Remarkably, the difference in toxicity could be mapped to the 10 amino acid segment of the A2-fragment that penetrates the central pore of the B-subunit pentamer. This region is responsible for maintaining A-/B-subunit interaction. A comparison of the *in vitro* stability of two hybrid toxins, differing only in this 10 amino acid segment, revealed that the Ctx A2 segment conferred a greater stability to the interaction between the A- and B-subunits than the corresponding segment from Etx A2. This suggests that the reason for the relative potency of Ctx compared with Etx stems from the increased ability of the A2-fragment of Ctx to maintain holotoxin stability during uptake and transport into intestinal epithelia. These findings highlight the importance of a region in the toxin that has hitherto been generally overlooked, and provides a possible contributory explanation for the difference in severity of cholera and traveller's diarrheal disease, respectively.

## CONCLUSION

Cholera toxin and related enterotoxins from *E. coli* continue to be a source of considerable fascination. While much is now known about the biogenesis of these toxins, the precise molecular basis by which the toxins are translocated across the bacterial outer membrane remains a challenging area for future investigation. The increasing insights being gained into the uptake and trafficking of the molecules in mammalian cells is also shedding light on aspects of eukaryotic cell biology. These toxins will therefore remain excellent models for investigating important cellular processes in both prokaryotic and eukaryotic systems.

## ACKNOWLEDGMENTS

We wish to thank the many colleagues and collaborators who have contributed to the many achievements documented in this review, particularly Lolke de Haan, Wayne Lencer, Simon Hardy, Jan Holmgren, Toufic Nashar, Neil Williams, Jun Yu, Chiara Rodighiero, Abu Tholib Aman, Sylvia Fraser, Jenny Rivett, and Arron Hearn. This was supported by a Research Development Grant from the University of Sydney.

## REFERENCES

Abendroth, J., Bagdasarian, M., Sandkvist, M. and Hol, W.G. (2004a). The Structure of the cytoplasmic domain of epsL, an inner membrane component of the type II secretion system of *Vibrio cholerae*: an unusual member of the actin-like ATPase superfamily. *J. Mol. Biol.*, **344,** 619–33.

Abendroth, J., Rice, A.E., McLuskey, K., Bagdasarian, M. and Hol, W. G. (2004b). The crystal structure of the periplasmic domain of the type II secretion system protein EpsM from *Vibrio cholerae*: the simplest version of the ferredoxin fold *J. Mol. Biol.*, **338,** 585–96.

Agbonlahor, D.E. and Odugbemi, T.O. (1982). Enteropathogenic, enterotoxigenic, and enteroinvasive *Escherichia coli* isolated from acute gastroenteritis patients in Lagos, Nigeria. *Trans. R. Soc. Trop. Med. Hyg.*, **76,** 265–7.

Albert, M.J., Siddique, A.K., Islam, M.S., Faruque, A.S., Ansaruzzaman, M., Faruque, S.M. and Sack, R.B. (1993). Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 in Bangladesh. *Lancet,* **341,** 704.

Aman, A.T., Fraser, S., Merritt, E.A., Rodigherio, C., Kenny, M., Ahn, M., Hol, W.G., Williams, N.A., Lencer, W.I. and Hirst, T.R. (2001). A mutant cholera toxin B subunit that binds GM1-ganglioside but lacks immunomodulatory or toxic activity. *Proc. Natl. Acad. Sci. USA,* **98,** 8536–41.

Angstrom, J., Teneberg, S. and Karlsson, K.A. (1994). Delineation and comparison of ganglioside-binding epitopes for the toxins of *Vibrio cholerae, Escherichia coli*, and *Clostridium tetani*: evidence for overlapping epitopes. *Proc. Natl. Acad. Sci. USA.* **91,** 11859–63.

Arnone, A., Bier, C.J., Cotton, F.A., Day, V.W., Hazen, E.E., Jr., Richardson, D.C., Yonath, A. and Richardson, J.S. (1971). A high-resolution structure of an inhibitor complex of the extracellular nuclease of *Staphylococcus aureus*. I. Experimental procedures and chain tracing. *J. Biol. Chem.* **246,** 2302–16.

Backstrom, M., Shahabi, V., Johansson, S., Teneberg, S., Kjellberg, A., Miller-Podraza, H., Holmgren, J. and Lebens, M. (1997). Structural basis for differential receptor binding of cholera and

*Escherichia coli* heat-labile toxins: influence of heterologous amino acid substitutions in the cholera B-subunit. *Mol. Microbiol.* **24,** 489–97.

Badizadegan, K., Wheeler, H.E., Fujinaga, Y. and Lencer, W.I. (2004). Trafficking of cholera toxin-ganglioside GM1 complex into Golgi and induction of toxicity depend on actin cytoskeleton. *Am. J. Physiol. Cell Physiol.* **287,** C1453–62.

Bardwell, J.C. (1994). Building bridges: disulphide bond formation in the cell *Mol. Microbiol.* **14,** 199–205.

Bardwell, J.C., McGovern, K. and Beckwith, J. (1991). Identification of a protein required for disulfide bond formation *in vivo. Cell*, **67,** 581–9.

Barlowe, C. (2000). Traffic COPs of the early secretory pathway. *Traffic,* **1,** 371–7.

Barua, D. (1991). In: *Cholera*, Vol. 1–35 (Eds. Baurua, D. and Greenough III, W. B.) Plenum, New York.

Baudry, B., Fasano, A., Ketley, J. and Kaper, J.B. (1992). Cloning of a gene (zot) encoding a new toxin produced by *Vibrio cholerae. Infect Immun.*, **60,** 428–34.

Black, R.E. (1986). In *Development of Vaccines and Drugs against Diarrhea* (Ed. Holmgren, R. M. J.), pp. 23–32.

Black, R.E., Merson, M.H., Rahman, A.S., Yunus, M., Alim, A.R., Huq, I., Yolken, R.H. and Curlin, G.T. (1980). A two-year study of bacterial, viral, and parasitic agents associated with diarrhea in rural Bangladesh. *J. Infect. Dis.*, **142,** 660–4.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature,* **349,** 117–27.

Boyd, E.F. and Waldor, M.K. (1999). Alternative mechanism of cholera toxin acquisition by *Vibrio cholerae*: generalized transduction of CTXPhi by bacteriophage CP-T1. *Infect. Immun.*, **67,** 5898–905.

Brickman, T.J., Boesman-Finkelstein, M., Finkelstein, R.A. and McIntosh, M.A. (1990). Molecular cloning and nucleotide sequence analysis of cholera toxin genes of the CtxA-*Vibrio cholerae* strain Texas Star-SR. *Infect. Immun.*, **58,** 4142–4.

Burch, R.M., Jelsema, C. and Axelrod, J. (1988). Cholera toxin and pertussis toxin stimulate prostaglandin E2 synthesis in a murine macrophage cell line. *J. Pharmacol. Exp. Ther.*, **244,** 765–73.

Champion, G.A., Neely, M.N., Brennan, M.A. and DiRita, V.J. (1997). A branch in the ToxR regulatory cascade of *Vibrio cholerae* revealed by characterization of *toxT* mutant strains. *Mol. Microbiol.*, **23,** 323–31.

Cheesman, C., Freedman, R.B. and Ruddock, L.W. (2004). The disassembly and reassembly of mutants of *Escherichia coli* heat-labile enterotoxin: replacement of proline 93 does not abolish the reassembly-competent and reassembly-incompetent states. *Biochemistry,* **43,** 1618–25.

Chen, A., Hu, T., Mikoryak, C. and Draper, R.K. (2002). Retrograde transport of protein toxins under conditions of COPI dysfunction. *Biochim. Biophys. Acta,* **1589,** 124–39.

Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J. and Smith, A.E. (1991). Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell,* **66,** 1027–36.

Cieplak, W., Jr., Mead, D. J., Messer, R.J. and Grant, C.C. (1995). Site-directed mutagenic alteration of potential active-site residues of the A subunit of *Escherichia coli* heat-labile enterotoxin. Evidence for a catalytic role for glutamic acid 112. *J. Biol. Chem.*, **270,** 30545–50.

Clements, J.D. and Finkelstein, R.A. (1979). Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. *Infect. Immun.*, **24,** 760–9.

Clements, J.D., Yancey, R.J. and Finkelstein, R.A. (1980). Properties of homogeneous heat-labile enterotoxin from *Escherichia coli. Infect. Immun.*, **29,** 91–7.

Cosson, P. and Letourneur, F. (1997). Coatomer (COPI)-coated vesicles: role in intracellular transport and protein sorting. *Curr. Opin. Cell Biol.*, **9,** 484–7.

Cuatrecasas, P. (1973). Gangliosides and membrane receptors for cholera toxin. *Biochemistry,* **12,** 3558–66.

Dallas, W.S. (1983). Conformity between heat-labile toxin genes from human and porcine enterotoxigenic *Escherichia coli. Infect. Immun.*, **40,** 647–52.

Dallas, W.S. and Falkow, S. (1979). The molecular nature of heat-labile enterotoxin (LT) of *Escherichia coli. Nature,* **277,** 406–7.

Dallas, W.S. and Falkow, S. (1980). Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature,* **288,** 499–501.

Dallas, W.S., Gill, D.M. and Falkow, S. (1979). Cistrons encoding *Escherichia coli* heat-labile toxin. *J. Bacteriol.*, **139,** 850–8.

Dams, E., De Wolf, M. and Dierick, W. (1991). Nucleotide sequence analysis of the CT operon of the *Vibrio cholerae* classical strain 569B. *Biochim. Biophys. Acta,* **1090,** 139–41.

Daniels, C.J., Bole, D.G., Quay, S.C. and Oxender, D.L. (1981). Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli. Proc. Natl. Acad. Sci. USA,* **78,** 5396–400.

Dartigalongue, C. and Raina, S. (1998). A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in *Escherichia coli. EMBO J.*, **17,** 3968–80.

Davis, B.M., Kimsey, H.H., Kane, A.V. and Waldor, M.K. (2002). A satellite phage-encoded antirepressor induces repressor aggregation and cholera toxin gene transfer. *EMBO J.*, **21,** 4240–9.

Davis, B.M., Lawson, E.H., Sandkvist, M., Ali, A., Sozhamannan, S. and Waldor, M.K. (2000). Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXphi. *Science,* **288,** 333–5.

de Mol, P., Brasseur, D., Hemelhof, W., Kalala, T., Butzler, J.P. and Vis, H.L. (1983). Enteropathogenic agents in children with diarrhea in rural Zaire. *Lancet,* **1,** 516–8.

De, S.N., Bhattacharya, K. and Sarkar, J.K. (1956). A study of the pathogenicity of strains of Bacterium coli from acute and chronic enteritis. *J. Pathol. Bacteriol.*, **71,** 201–9.

Denning, G.M., Ostedgaard, L.S., Cheng, S.H., Smith, A.E. and Welsh, M.J. (1992). Localization of cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia. *J. Clin. Invest.*, **89,** 339–49.

DiRita, V.J. (1992). Coordinate expression of virulence genes by ToxR in *Vibrio cholerae. Mol. Microbiol.*, **6,** 451–8.

DiRita, V.J. and Mekalanos, J.J. (1991). Periplasmic interaction between two membrane regulatory proteins, ToxR and ToxS, results in signal transduction and transcriptional activation. *Cell,* **64,** 29–37.

DiRita, V.J., Neely, M., Taylor, R.K. and Bruss, P.M. (1996). Differential expression of the ToxR regulon in classical and E1 Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over toxT expression. *Proc. Natl. Acad. Sci. USA,* **93,** 7991–5.

Driessen, A.J. (1992). Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force. *EMBO J,* **11,** 847–53.

Driessen, A.J., Fekkes, P. and van der Wolk, J.P. (1998). The Sec system. *Curr. Opin. Microbiol.*, **1,** 216–22.

Driessen, A.J. and Wickner, W. (1991). Proton transfer is rate-limiting for translocation of precursor proteins by the *Escherichia coli* translocase. *Proc. Natl. Acad. Sci. USA,* **88,** 2471–5.

D'Souza-Schorey, C., Li, G., Colombo, M.I. and Stahl, P.D. (1995). A regulatory role for ARF6 in receptor-mediated endocytosis. *Science,* **267,** 1175–8.

Dykes, C.W., Halliday, I.J., Hobden, A.N., Read, M.J. and Harford, S. (1985). A comparison of the nucleotide sequence of the A subunit of heat-labile enterotoxin and cholera toxin. *FEMS Microbiol. Lett.*, **26,** 171–4.

Echeverria, P., Seriwatana, J., Taylor, D.N., Yanggratoke, S. and Tirapat, C. (1985). A comparative study of enterotoxigenic *Escherichia coli*, Shigella, Aeromonas, and Vibrio as etiologies of diarrhea in northeastern Thailand. *Am. J. Trop. Med. Hyg.*, **34,** 547–54.

Evans, D.G., Olarte, J., DuPont, H.L., Evans, D.J., Jr., Galindo, E., Portnoy, B.L. and Conklin, R.H. (1977). Enteropathogens associated with pediatric diarrhea in Mexico City. *J. Pediatr.*, **91,** 65–8.

Faruque, S.M., Asadulghani, Kamruzzaman, M., Nandi, R.K., Ghosh, A.N., Nair, G.B., Mekalanos, J.J. and Sack, D.A. (2002). RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXphi. *Infect. Immun.*, **70,** 163–70.

Fasano, A., Baudry, B., Pumplin, D.W., Wasserman, S.S., Tall, B.D., Ketley, J.M. and Kaper, J.B. (1991). *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc. Natl. Acad. Sci. USA,* **88,** 5242–6.

Field, M., Fromm, D., al-Awqati, Q. and Greenough, W.B., 3rd (1972). Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. *J. Clin. Invest.*, **51,** 796–804.

Field, M., Rao, M.C. and Chang, E.B. (1989). Intestinal electrolyte transport and diarrheal disease (1). *N. Engl. J. Med.*, **321,** 800–6.

Finkelstein, R.A., Atthasampunna, P., Chulasamaya, M. and Charunmethee, P. (1966). Pathogenesis of experimental cholera: biologic ativities of purified procholeragen A. *J. Immunol.*, **96,** 440–9.

Fitts, R., Reuveny, Z., van Amsterdam, J., Mulholland, J. and Botstein, D. (1987). Substitution of tyrosine for either cysteine in beta-lactamase prevents release from the membrane during secretion. *Proc. Natl. Acad. Sci. USA,* **84,** 8540–3.

Fraser, S.A., de Haan, L., Hearn, A.R., Bone, H.K., Salmond, R.J., Rivett, A.J., Williams, N.A. and Hirst, T.R. (2003). Mutant *Escherichia coli* heat-labile toxin B subunit that separates toxoid-mediated signaling and immunomodulatory action from trafficking and delivery functions. *Infect. Immun.*, **71,** 1527–37.

Fukuta, S., Magnani, J.L., Twiddy, E.M., Holmes, R.K. and Ginsburg, V. (1988). Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infect. Immun.*, **56,** 1748–53.

Geller, B.L. (1991). Energy requirements for protein translocation across the *Escherichia coli* inner membrane. *Mol. Microbiol.*, **5,** 2093–8.

Gennaro, M.L. and Greenaway, P.J. (1983). Nucleotide sequences within the cholera toxin operon. *Nucleic Acids Res.*, **11,** 3855–61.

Gennaro, M.L., Greenaway, P.J. and Broadbent, D.A. (1982). The expression of biologically active cholera toxin in *Escherichia coli*. *Nucleic Acids Res.*, **10,** 4883–90.

Gill, D.M. (1975). Involvement of nicotinamide adenine dinucleotide in the action of cholera toxin *in vitro*. *Proc. Natl. Acad. Sci. USA,* **72,** 2064–8.

Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T. and Pepperkok, R. (1999). Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nat. Cell Biol.*, **1,** 423–30.

Goldberg, I. and Mekalanos, J.J. (1986). Effect of a recA mutation on cholera toxin gene amplification and deletion events. *J. Bacteriol.*, **165,** 723–31.

Griffiths, G., Ericsson, M., Krijnse-Locker, J., Nilsson, T., Goud, B., Soling, H.D., Tang, B.L., Wong, S.H. and Hong, W. (1994). Localization of the Lys, Asp, Glu, Leu tetrapeptide receptor to the Golgi complex and the intermediate compartment in mammalian cells. *J. Cell Biol.*, **127,** 1557–74.

Guidry, J.J., Cardenas, L., Cheng, E. and Clements, J.D. (1997). Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun.*, **65,** 4943–50.

Gyles, C.L., Palchaudhuri, S. and Maas, W.K. (1977). Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. *Science,* **198,** 198–9.

Halm, D.R., Rechkemmer, G.R., Schoumacher, R.A. and Frizzell, R.A. (1988). Apical membrane chloride channels in a colonic cell line activated by secretory agonists. *Am. J. Physiol.*, **254,** C505–11.

Hardy, S.J. and Hedges, P.A. (1996). Reduced B subunit of heat-labile enterotoxin associates with membranes *in vivo*. *Eur. J. Biochem.*, **236,** 412–8.

Hardy, S.J., Holmgren, J., Johansson, S., Sanchez, J. and Hirst, T.R. (1988). Coordinated assembly of multisubunit proteins: oligomerization of bacterial enterotoxins *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA,* **85,** 7109–13.

Hase, C.C. and Mekalanos, J.J. (1998). TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA,* **95,** 730–4.

Hayano, T., Takahashi, N., Kato, S., Maki, N. and Suzuki, M. (1991). Two distinct forms of peptidylprolyl-cis-trans-isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. *Biochemistry,* **30,** 3041–8.

Hazes, B. and Read, R.J. (1997). Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry,* **36,** 11051–4.

Hengge, R. and Boos, W. (1985). Defective secretion of maltose- and ribose-binding proteins caused by a truncated periplasmic protein in *Escherichia coli*. *J. Bacteriol.*, **162,** 972–8.

Hepler, J.R. and Gilman, A.G. (1992). G proteins. *Trends Biochem. Sci.*, **17,** 383–7.

Hirst, T.R., Hillary, J.B., Ruddock, L.W. and Yu, J. (1995). Translocation of folded proteins across bacterial outer membranes: a novel secretory phenomenon. *Biochem. Soc. Trans.*, **23,** 985–91.

Hirst, T. R. and Leece, R. (1991). The phenomenon of toxin secretion by vibrios and aeromonads. *Experientia,* **47,** 429–31.

Hirst, T.R. and Holmgren, J. (1987a). Conformation of protein secreted across bacterial outer membranes: a study of enterotoxin translocation from *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA,* **84,** 7418–22.

Hirst, T.R. and Holmgren, J. (1987b). Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae*. *J. Bacteriol.*, **169,** 1037–45.

Hirst, T.R., Randall, L.L. and Hardy, S.J. (1984). Cellular location of heat-labile enterotoxin in *Escherichia coli*. *J. Bacteriol.*, **157,** 637–42.

Hirst, T.R., Hardy, S.J. and Randall, L.L. (1983). Assembly *in vivo* of enterotoxin from *Escherichia coli*: formation of the B subunit oligomer. *J. Bacteriol.*, **153,** 21–6.

Hofstra, H. and Witholt, B. (1984). Kinetics of synthesis, processing, and membrane transport of heat-labile enterotoxin, a periplasmic protein in *Escherichia coli*. *J. Biol. Chem.*, **259,** 15182–7.

Hol, W.G.J., Sixma, T.K., and Merritt, E.A. (1995). In: *Bacterial toxins and Virulence Factors in Disease.* (Ed. Tu, A.T.), pp. 185–223. Marcel Dekker, New York.

Holmgren, J. (1973). Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoid. *Infect. Immun.*, **8,** 851–9.

Horstman, A.L., Bauman, S.J. and Kuehn, M.J. (2004). Lipopolysaccharide 3-deoxy-D-manno-octulosonic acid (Kdo) core determines bacterial association of secreted toxins. *J. Biol. Chem.*, **279,** 8070–5.

Horstman, A.L. and Kuehn, M.J. (2002). Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway. *J. Biol. Chem.*, **277,** 32538–45.

Huber, K.E. and Waldor, M.K. (2002). Filamentous phage integration requires the host recombinases XerC and XerD. *Nature,* **417,** 656–9.

Inoue, T., Tsuji, T., Koto, M., Imamura, S. and Miyama, A. (1993). Amino acid sequence of heat-labile enterotoxin from chicken enterotoxigenic *Escherichia coli* is identical to that of human strain H 10407. *FEMS Microbiol. Lett.*, **108,** 157–61.

Ito, K. and Beckwith, J.R. (1981). Role of the mature protein sequence of maltose-binding protein in its secretion across the *E. coli* cytoplasmic membrane. *Cell,* **25,** 143–50.

Jaenicke, R. (1987). Folding and association of proteins. *Prog. Biophys. Mol. Biol.*, **49,** 117–237.

Jaenicke, R. (1991). Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry,* **30,** 3147–61.

Jobling, M.G. and Holmes, R.K. (1991). Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis. *Mol. Microbiol.*, **5,** 1755–67.

Jobling, M.G. and Holmes, R.K. (2000). Identification of motifs in cholera toxin A1 polypeptide that are required for its interaction with human ADP-ribosylation factor 6 in a bacterial two-hybrid system. *Proc. Natl. Acad. Sci. USA,* **97,** 14662–7.

Kahn, R.A. and Gilman, A.G. (1986). The protein cofactor necessary for ADP-ribosylation of Gs by cholera toxin is itself a GTP binding protein. *J. Biol. Chem.*, **261,** 7906–11.

Kamitani, S., Akiyama, Y. and Ito, K. (1992). Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. *EMBO J.*, **11,** 57–62.

Karaolis, D.K., Johnson, J.A., Bailey, C.C., Boedeker, E.C., Kaper, J.B. and Reeves, P.R. (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proc. Natl. Acad. Sci. USA,* **95,** 3134–9.

Karlsson, K.A., Teneberg, S., Angstrom, J., Kjellberg, A., Hirst, T.R., Berstrom, J. and Miller-Podraza, H. (1996). Unexpected carbohydrate cross-binding by *Escherichia coli* heat-labile enterotoxin. Recognition of human and rabbit target cell glycoconjugates in comparison with cholera toxin. *Bioorg. Med. Chem.*, **4,** 1919–28.

Kesty, N.C., Mason, K.M., Reedy, M., Miller, S.E. and Kuehn, M.J. (2004). Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells. *EMBO J.*, **23,** 4538–49.

Kimberg, D.V., Field, M., Johnson, J., Henderson, A. and Gershon, E. (1971). Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.*, **50,** 1218–30.

Koch, R. (1884). An address on cholera and its bacillus. *Br. Med. J.*, **2,** 403–407.

Kopito, R.R. (1997). ER quality control: the cytoplasmic connection. *Cell,* **88,** 427–30.

Koshland, D. and Botstein, D. (1982). Evidence for posttranslational translocation of beta-lactamase across the bacterial inner membrane. *Cell,* **30,** 893–902.

Kovacikova, G., Lin, W. and Skorupski, K. (2004). *Vibrio cholerae* AphA uses a novel mechanism for virulence gene activation that involves interaction with the LysR-type regulator AphB at the tcpPH promoter. *Mol. Microbiol.*, **53,** 129–42.

Kovacikova, G. and Skorupski, K. (1999). A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the tcpPH promoter to activate expression of the ToxR virulence cascade. *J. Bacteriol.*, **181,** 4250–6.

Kuziemko, G.M., Stroh, M. and Stevens, R.C. (1996). Cholera toxin binding affinity and specificity for gangliosides determined by surface plasmon resonance. *Biochemistry,* **35,** 6375–84.

Lang, K., Schmid, F.X. and Fischer, G. (1987). Catalysis of protein folding by prolyl isomerase. *Nature,* **329,** 268–70.

Lauer, S., Goldstein, B., Nolan, R.L. and Nolan, J.P. (2002). Analysis of cholera toxin-ganglioside interactions by flow cytometry. *Biochemistry,* **41,** 1742–51.

Lee, S.H., Butler, S.M. and Camilli, A. (2001). Selection for *in vivo* regulators of bacterial virulence. *Proc. Natl. Acad. Sci. USA,* **98,** 6889–94.

Lencer, W.I., Constable, C., Moe, S., Jobling, M.G., Webb, H.M., Ruston, S., Madara, J.L., Hirst, T.R. and Holmes, R.K. (1995). Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell. Biol.*, **131,** 951–62.

Lencer, W.I., Delp, C., Neutra, M.R. and Madara, J.L. (1992). Mechanism of cholera toxin action on a polarized human intestinal epithelial cell line: role of vesicular traffic. *J. Cell Biol.*, **117,** 1197–1209.

Lencer, W.I. and Tsai, B. (2003). The intracellular voyage of cholera toxin: going retro. *Trends Biochem. Sci.*, **28,** 639–45.

Leong, J., Vinal, A.C. and Dallas, W.S. (1985). Nucleotide sequence comparison between heat-labile toxin B-subunit cistrons from *Escherichia coli* of human and porcine origin. *Infect. Immun.*, **48,** 73–7.

Lesieur, C., Cliff, M.J., Carter, R., James, R.F., Clarke, A.R. and Hirst, T.R. (2002). A kinetic model of intermediate formation during assembly of cholera toxin B-subunit pentamers. *J. Biol. Chem.*, **277,** 16697–704.

Liu, J. and Walsh, C.T. (1990). Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proc. Natl. Acad. Sci. USA,* **87,** 4028–32.

Lockman, H. and Kaper, J.B. (1983). Nucleotide sequence analysis of the A2 and B subunits of *Vibrio cholerae* enterotoxin. *J. Biol. Chem.*, **258,** 13722–6.

London, E. and Luongo, C.L. (1989). Domain-specific bias in arginine/lysine usage by protein toxins. *Biochem. Biophys. Res. Commun.*, **160,** 333–9.

Majoul, I., Sohn, K., Wieland, F.T., Pepperkok, R., Pizza, M., Hillemann, J. and Soling, H.D. (1998). KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. *J. Cell Biol.*, **143,** 601–12.

Massol, R.H., Larsen, J.E., Fujinaga, Y., Lencer, W.I. and Kirchhausen, T. (2004). Cholera toxin toxicity does not require functional Arf6- and dynamin-dependent endocytic pathways. *Mol. Biol. Cell,* **15,** 3631–41.

Matlack, K.E., Mothes, W. and Rapoport, T.A. (1998). Protein translocation: tunnel vision. *Cell,* **92,** 381–90.

Matthews, J.B., Awtrey, C.S. and Madara, J.L. (1992). Microfilament-dependent activation of Na+/K+/2Cl-cotransport by cAMP in intestinal epithelial monolayers. *J. Clin. Invest.*, **90,** 1608–13.

McLeod, S.M. and Waldor, M.K. (2004). Characterization of XerC- and XerD-dependent CTX phage integration in *Vibrio cholerae*. *Mol. Microbiol.*, **54,** 935–47.

McRoberts, J.A., Beuerlein, G. and Dharmsathaphorn, K. (1985). Cyclic AMP and Ca2+-activated K+ transport in a human colonic epithelial cell line. *J. Biol. Chem.*, **260,** 14163–72.

Mekalanos, J.J. (1983). Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell,* **35,** 253–63.

Mekalanos, J.J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.*, **174,** 1–7.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D., Harford, N., Groyne, F. and de Wilde, M. (1983). Cholera toxin genes: nucleotide sequence, deletion analysis, and vaccine development. *Nature,* **306,** 551–7.

Merritt, E.A., Kuhn, P., Sarfaty, S., Erbe, J.L., Holmes, R.K. and Hol, W.G. (1998). The 1.25 A resolution refinement of the cholera toxin B-pentamer: evidence of peptide backbone strain at the receptor-binding site. *J. Mol. Biol.*, **282,** 1043–59.

Merritt, E.A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J.A. and Hol, W.G. (1994). Crystal structure of cholera toxin B-pentamer bound to receptor GM1 pentasaccharide. *Protein Sci.*, **3,** 166–75.

Merz, A.J., So, M. and Sheetz, M.P. (2000). Pilus retraction powers bacterial twitching motility. *Nature,* **407,** 98–102.

Miller, V.L., DiRita, V.J. and Mekalanos, J.J. (1989) Identification of toxS, a regulatory gene whose product enhances toxR-mediated activation of the cholera toxin promoter. *J. Bacteriol.*, **171,** 1288–93.

Miller, V.L. and Mekalanos, J.J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. *Proc. Natl. Acad. Sci. USA,* **81,** 3471–5.

Miller, V.L. and Mekalanos, J.J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *J. Bacteriol.*, **170,** 2575–83.

Miller, V.L., Taylor, R.K. and Mekalanos, J.J. (1987). Cholera toxin transcriptional activator toxR is a transmembrane DNA binding protein. *Cell,* **48,** 271–9.

Minsky, A., Summers, R.G. and Knowles, J.R. (1986). Secretion of beta-lactamase into the periplasm of *Escherichia coli*: evidence for a distinct release step associated with a conformational change. *Proc. Natl. Acad. Sci. USA,* **83,** 4180–4.

Missiakas, D. and Raina, S. (1997). Protein folding in the bacterial periplasm. *J. Bacteriol.*, **179,** 2465–71.

Moss, J., Manganiello, V.C. and Vaughan, M. (1976). Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A protomer: possible role in the activation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA,* **73,** 4424–7.

Moss, J., Stanley, S.J., Vaughan, M. and Tsuji, T. (1993). Interaction of ADP-ribosylation factor with *Escherichia coli* enterotoxin that contains an inactivating lysine 112 substitution. *J. Biol. Chem.*, **268,** 6383–7.

Moss, J. and Vaughan, M. (1995). Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. *J. Biol. Chem.*, **270,** 12327–30.

Munro, S. and Pelham, H.R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell,* **48,** 899–907.

Murzin, A.G. (1993). OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences. *EMBO J.*, **12,** 861–7.

Nakashima, K., Eguchi, Y. and Nakasone, N. (1995). Characterization of an enterotoxin produced by *Vibrio cholerae* O139. *Microbiol. Immunol.*, **39,** 87–94.

Nashar, T.O., Williams, N.A. and Hirst, T.R. (1998). Importance of receptor binding in the immunogenicity, adjuvanticity, and therapeutic properties of cholera toxin and *Escherichia coli* heat-labile enterotoxin. *Med. Microbiol. Immunol. (Berl),* **187,** 3–10.

Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli. Clin. Microbiol. Rev.*, **11,** 142–201.

Nichols, B.J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol.*, **4,** 374–8.

Nichols, B.J. and Lippincott-Schwartz, J. (2001). Endocytosis without clathrin coats. *Trends Cell Biol.*, **11,** 406–12.

Nye, M.B., Pfau, J.D., Skorupski, K. and Taylor, R.K. (2000). *Vibrio cholerae* H-NS silences virulence gene expression at multiple steps in the ToxR regulatory cascade. *J. Bacteriol.*, **182,** 4295–303.

Nye, M.B. and Taylor, R.K. (2003). *Vibrio cholerae* H-NS domain structure and function with respect to transcriptional repression of ToxR regulon genes reveals differences among H-NS family members. *Mol. Microbiol.*, **50,** 427–44.

O'Neal, C.J., Amaya, E.I., Jobling, M.G., Holmes, R.K. and Hol, W.G. (2004). Crystal structures of an intrinsically active cholera toxin mutant yield insight into the toxin activation mechanism. *Biochemistry,* **43,** 3772–82.

Orci, L., Perrelet, A., Ravazzola, M., Amherdt, M., Rothman, J.E. and Schekman, R. (1994). Coatomer-rich endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA,* **91,** 11924–8.

Orlandi, P.A., Critchley, D.R. and Fishman, P.H. (1994). The heat-labile enterotoxin of *Escherichia coli* binds to polylactosaminoglycan-containing receptors in CaCo-2 human intestinal epithelial cells. *Biochemistry,* **33,** 12886–95.

Orlandi, P.A. and Fishman, P.H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.*, **141,** 905–15.

Pacuszka, T. and Fishman, P.H. (1991). Metabolism of cholesterol, phosphatidylethanolamine, and stearylamine analogues of GM1 ganglioside by rat glioma C6 cells. *Biochim. Biophys. Acta,* **1083,** 153–60.

Palva, E.T., Hirst, T.R., Hardy, S.J., Holmgren, J. and Randall, L. (1981). Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli. J. Bacteriol.*, **146,** 325–30.

Parton, R.G. (1994). Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J. Histochem. Cytochem.*, **42,** 155–66.

Pearson, G.D., Woods, A., Chiang, S.L. and Mekalanos, J.J. (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. *Proc. Natl. Acad. Sci. USA,* **90,** 3750–4.

Peek, J.A. and Taylor, R.K. (1992). Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae. Proc. Natl. Acad. Sci. USA,* **89,** 6210–4.

Pelham, H.R. (1988). Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO J.*, **7,** 913–8.

Peterson, J.W. and Ochoa, L.G. (1989). Role of prostaglandins and cAMP in the secretory effects of cholera toxin. *Science,* **245,** 857–9.

Picciotto, M.R., Cohn, J.A., Bertuzzi, G., Greengard, P. and Nairn, A.C. (1992). Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.*, **267,** 12742–52.

Powell, D.W. (1974). Intestinal conductance and permselectivity changes with theophylline and choleragen. *Am. J. Physiol.*, **227,** 1436–43.

Pugsley, A.P. (1993). The complete general secretory pathway in Gram-negative bacteria. *Microbiol. Rev.*, **57,** 50–108.

Pugsley, A.P., Francetic, O., Possot, O.M., Sauvonnet, N. and Hardie, K.R. (1997). Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria—a review. *Gene,* **192,** 13–9.

Radhakrishna, H. and Donaldson, J.G. (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J. Cell Biol.*, **139,** 49–61.

Radhakrishna, H., Klausner, R.D. and Donaldson, J.G. (1996). Aluminum fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. *J. Cell Biol.*, **134,** 935–47.

Randall, L.L. and Hardy, S.J. (1989). Unity in function in the absence of consensus in sequence: role of leader peptides in export. *Science,* **243,** 1156–9.

Robien, M.A., Krumm, B.E., Sandkvist, M. and Hol, W.G. (2003). Crystal structure of the extracellular protein secretion NTPase EpsE of *Vibrio cholerae. J. Mol. Biol.*, **333,** 657–74.

Rodighiero, C., Aman, A.T., Kenny, M.J., Moss, J., Lencer, W.I. and Hirst, T.R. (1999). Structural basis for the differential toxicity of cholera toxin and *Escherichia coli* heat-labile enterotoxin. Construction of hybrid toxins identifies the A2-domain as the determinant of differential toxicity. *J. Biol. Chem.*, **274,** 3962–9.

Rodighiero, C., Tsai, B., Rapoport, T.A. and Lencer, W.I. (2002). Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. *EMBO Rep.*, **3,** 1222–7.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol.*, **279,** 485–99.

Sack, R.B., Albert, M.J. and Siddique, A.K. (1996). Emergence of *Vibrio cholerae* O139. *Curr. Clin. Top Infect. Dis.*, **16,** 172–93.

Sanchez, J. and Holmgren, J. (1989). Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA,* **86,** 481–5.

Sandkvist, M. (2001a). Biology of type II secretion. *Mol. Microbiol.*, **40,** 271–83.

Sandkvist, M. (2001b). Type II secretion and pathogenesis. *Infect. Immun.*, **69,** 3523–35.

Sandkvist, M., Bagdasarian, M. and Howard, S.P. (2000). Characterization of the multimeric Eps complex required for cholera toxin secretion. *Int. J. Med. Microbiol.*, **290,** 345–50.

Sandkvist, M., Hough, L.P., Bagdasarian, M.M. and Bagdasarian, M. (1999). Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholerae. J. Bacteriol.*, **181,** 3129–35.

Sandkvist, M., Bagdasarian, M., Howard, S.P. and DiRita, V. J. (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae. EMBO J.*, **14,** 1664–73.

Sandkvist, M., Hirst, T.R. and Bagdasarian, M. (1990). Minimal deletion of amino acids from the carboxyl terminus of the B subunit of heat-labile enterotoxin causes defects in its assembly and release from the cytoplasmic membrane of *Escherichia coli. J. Biol. Chem.*, **265,** 15239–44.

Sandkvist, M., Hirst, T.R. and Bagdasarian, M. (1987). Alterations at the carboxyl terminus change assembly and secretion properties of the B subunit of *Escherichia coli* heat-labile enterotoxin. *J. Bacteriol.*, **169,** 4570–6.

Sandvig, K., Garred, O. and van Deurs, B. (1996). Thapsigargin-induced transport of cholera toxin to the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA,* **93,** 12339–43.

Sandvig, K., Ryd, M., Garred, O., Schweda, E., Holm, P.K. and van Deurs, B. (1994). Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga B-fragment is regulated by butyric acid and cAMP. *J. Cell Biol.*, **126,** 53–64.

Schmitz, A., Herrgen, H., Winkeler, A. and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol.*, **148,** 1203–12.

Shapiro, M., Matthews, J., Hecht, G., Delp, C. and Madara, J.L. (1991). Stabilization of F-actin prevents cAMP-elicited Cl-secretion in T84 cells. *J. Clin. Invest.*, **87,** 1903–9.

Simonen, M. and Palva, I. (1993). Protein secretion in Bacillus species. *Microbiol. Rev.*, **57,** 109–37.

Sixma, T.K., Kalk, K.H., van Zanten, B.A., Dauter, Z., Kingma, J., Witholt, B. and Hol, W.G. (1993). Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J. Mol. Biol.*, **230,** 890–918.

Sixma, T.K., Pronk, S.E., Kalk, K.H., van Zanten, B.A. Berghuis, A.M. and Hol, W.G. (1992). Lactose binding to heat-labile enterotoxin revealed by X-ray crystallography. *Nature*, **355,** 561–4.

Sixma, T.K., Pronk, S.E., Kalk, K.H., Wartna, E.S., van Zanten, B.A., Witholt, B. and Hol, W.G. (1991). Crystal structure of a cholera toxin-related heat-labile enterotoxin from E. coli. *Nature*, **351,** 371–7.

Skorupski, K. and Taylor, R.K. (1999). A new level in the *Vibrio cholerae* ToxR virulence cascade: AphA is required for transcriptional activation of the tcpPH operon. *Mol. Microbiol.*, **31,** 763–71.

Smith, H.W. and Linggood, M.A. (1971). The transmissible nature of enterotoxin production in a human enteropathogenic strain of *Escherichia coli. J. Med. Microbiol.*, **4,** 301–5.

So, M., Dallas, W.S. and Falkow, S. (1978). Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. *Infect. Immun.*, **21,** 405–11.

Speelman, P., Rabbani, G.H., Bukhave, K. and Rask-Madsen, J. (1985). Increased jejunal prostaglandin E2 concentrations in patients with acute cholera. *Gut*, **26,** 188–93.

Spicer, E.K. and Noble, J.A. (1982). *Escherichia coli* heat-labile enterotoxin. Nucleotide sequence of the A subunit gene. *J. Biol. Chem.*, **257,** 5716–21.

Stamnes, M.A., Craighead, M.W., Hoe, M.H., Lampen, N., Geromanos, S., Tempst, P. and Rothman, J.E. (1995). An integral membrane component of coatomer-coated transport vesicles defines a family of proteins involved in budding. *Proc. Natl. Acad. Sci. USA,* **92,** 8011–5.

Steffen, R. (1986). Epidemiologic studies of travelers' diarrhea, severe gastrointestinal infections, and cholera. *Rev. Infect. Dis.*, **8 Suppl 2,** S122–30.

Takao, T., Tominaga, N., Yoshimura, S., Shimonishi, Y., Hara, S., Inoue, T. and Miyama, A. (1985). Isolation, primary structure and synthesis of heat-stable enterotoxin produced by *Yersinia enterocolitica. Eur. J. Biochem.*, **152,** 199–206.

Tang, B.L., Wong, S.H., Qi, X.L., Low, S.H. and Hong, W. (1993). Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. *J. Cell. Biol.*, **120,** 325–8.

Teneberg, S., Hirst, T.R., Angstrom, J. and Karlsson, K.A. (1994). Comparison of the glycolipid-binding specificities of cholera toxin and porcine *Escherichia coli* heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. *Glycoconj. J.*, **11,** 533–40.

Teter, K., Allyn, R.L., Jobling, M.G. and Holmes, R.K. (2002). Transfer of the cholera toxin A1 polypeptide from the endoplasmic reticulum to the cytosol is a rapid process facilitated by the endoplasmic reticulum-associated degradation pathway. *Infect. Immun.*, **70,** 6166–71.

Teter, K. and Holmes, R.K. (2002). Inhibition of endoplasmic reticulum-associated degradation in CHO cells resistant to cholera toxin, Pseudomonas aeruginosa exotoxin A, and ricin. *Infect. Immun.*, **70,** 6172–9.

Thielman, N.M., Marcinkiewicz, M., Sarosiek, J., Fang, G.D. and Guerrant, R.L. (1997). Role of platelet-activating factor in Chinese hamster ovary cell responses to cholera toxin. *J. Clin. Invest.*, **99,** 1999–2004.

Tischler, A.D., Lee, S.H. and Camilli, A. (2002). The *Vibrio cholerae* vieSAB locus encodes a pathway contributing to cholera toxin production. *J. Bacteriol.*, **184,** 4104–13.

Tomb, J.F. (1992). A periplasmic protein disulfide oxidoreductase is required for transformation of Haemophilus influenzae Rd. *Proc. Natl. Acad. Sci. USA,* **89,** 10252–6.

Torgersen, M.L., Skretting, G., van Deurs, B. and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci.*, **114,** 3737–47.

Tousson, A., Fuller, C.M. and Benos, D.J. (1996). Apical recruitment of CFTR in T-84 cells is dependent on cAMP and microtubules but not Ca2+ or microfilaments. *J. Cell Sci.*, **109 (Pt 6),** 1325–34.

Trucksis, M., Galen, J.E., Michalski, J., Fasano, A. and Kaper, J.B. (1993). Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. *Proc. Natl. Acad. Sci. USA,* **90,** 5267–71.

Tsai, B. and Rapoport, T.A. (2002). Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol.*, **159,** 207–16.

Tsai, B., Rodighiero, C., Lencer, W.I. and Rapoport, T.A. (2001). Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell,* **104,** 937–48.

Tsuchiya, M., Price, S.R., Tsai, S.C., Moss, J. and Vaughan, M. (1991). Molecular identification of ADP-ribosylation factor mRNAs and their expression in mammalian cells. *J. Biol. Chem.*, **266,** 2772–7.

Tsuji, T., Honda, T., Miwatani, T., Wakabayashi, S. and Matsubara, H. (1985). Analysis of receptor-binding site in *Escherichia coli* enterotoxin. *J. Biol. Chem.*, **260,** 8552–8.

van den Akker, F., Merritt, E.A., Pizza, M., Domenighini, M., Rappuoli, R. and Hol, W. G. (1995). The Arg7Lys mutant of heat-labile enterotoxin exhibits great flexibility of active site loop 47-56 of the A subunit. *Biochemistry,* **34,** 10996–1004.

Van Dop, C., Tsubokawa, M., Bourne, H.R. and Ramachandran, J. (1984). Amino acid sequence of retinal transducin at the site ADP-ribosylated by cholera toxin. *J. Biol. Chem.*, **259,** 696–8.

Van Heyningen, W.E., Carpenter, C.C., Pierce, N.F. and Greenough, W. B., 3rd (1971). Deactivation of cholera toxin by ganglioside. *J. Infect. Dis.*, **124,** 415–8.

Waldman, R.J. (1998). Cholera vaccination in refugee settings. *Jama,* **279,** 552–3.

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science,* **272,** 1910–4.

Waldor, M.K., Rubin, E.J., Pearson, G.D., Kimsey, H. and Mekalanos, J.J. (1997). Regulation, replication, and integration functions of the *Vibrio cholerae* CTXphi are encoded by region RS2. *Mol. Microbiol.*, **24,** 917–26.

Werner, E.D., Brodsky, J.L. and McCracken, A.A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc. Natl. Acad. Sci. USA,* **93,** 13797–801.

White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. and Stelzer, E.H. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol.*, **147,** 743–60.

Wolf, A.A., Fujinaga, Y. and Lencer, W.I. (2002). Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem.*, **277,** 16249–56.

Wolf, A.A., Jobling, M.G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J.L., Holmes, R.K. and Lencer, W.I. (1998). Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell Biol.*, **141,** 917–27.

Wolfgang, M., van Putten, J.P., Hayes, S.F., Dorward, D. and Koomey, M. (2000). Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J.*, **19,** 6408–18.

Xia, Q.C., Chang, D., Blacher, R. and Lai, C.Y. (1984). The primary structure of the COOH-terminal half of cholera toxin subunit A1 containing the ADP-ribosylation site. *Arch. Biochem. Biophys.*, **234,** 363–70.

Yamamoto, T., Gojobori, T. and Yokota, T. (1987). Evolutionary origin of pathogenic determinants in enterotoxigenic *Escherichia coli* and *Vibrio cholerae* O1. *J. Bacteriol.*, **169,** 1352–7.

Yamamoto, T., Tamura, T. and Yokota, T. (1984). Primary structure of heat-labile enterotoxin produced by *Escherichia coli* pathogenic for humans. *J. Biol. Chem.*, **259,** 5037–44.

Yamamoto, T. and Yokota, T. (1982). Release of heat-labile enterotoxin subunits by *Escherichia coli. J. Bacteriol.*, **150,** 1482–4.

Yamamoto, T. and Yokota, T. (1983). Plasmids of enterotoxigenic *Escherichia coli* H10407: evidence for two heat-stable enterotoxin genes and a conjugal transfer system. *J. Bacteriol.*, **153,** 1352–60.

Yu, J., Webb, H. and Hirst, T.R. (1992) A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae. Mol. Microbiol.*, **6,** 1949–58.

Zhang, R.G., Scott, D.L., Westbrook, M.L., Nance, S., Spangler, B.D., Shipley, G.G. and Westbrook, E.M. (1995). The three-dimensional crystal structure of cholera toxin. *J. Mol. Biol.*, **251,** 563–73.

CHAPTER

# 16

# *Bordetella* protein toxins

*Jiri Masin, Peter Sebo, and Camille Locht*

## INTRODUCTION

The genus *Bordetella* comprises several species, three of which, *Bordetella pertussis, Bordetella parapertussis*, and *Bordetella bronchiseptica,* are important pathogens of mammalian hosts. *Bordetella avium* is a bird pathogen, whereas the remaining *Bordetella* species are rarely isolated from clinical specimens, although some of them may be opportunistic pathogens. The best-studied species are *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*, and their genome sequences are known (Parkhill *et al.*, 2003). *B. pertussis* is the causative agent of whooping cough, and *B. parapertussis* causes a milder whooping cough–like syndrome in humans. *B. bronchiseptica* is essentially a swine and dog pathogen and causes atrophic rhinitis in pigs, often associated with a co-infection by *Pasteurella multocida*. However, *B. bronchiseptica* is also relatively frequently isolated from patients suffering from acquired immunodeficiency, whereas *B. pertussis* is rarely found in immuno-compromised individuals.

The pathogenic potential of the *Bordetella* species relies on multiple factors, including several adhesins and toxins (Locht, 1999). Genomic studies have revealed an even more complex array of different virulence factors (Locht *et al.*, 2001), most of which are under the control of a two-component transcriptional regulatory system named BvgA/S. In addition to three major protein toxins, *Bordetella* produces non-protein toxins, such as endotoxin or lipopolysaccharide and tracheal cytotoxin. These toxins may sometimes act in synergy with the protein toxins. The three major protein toxins are pertussis toxin, adenylate cyclase, and dermonecrotic toxin, and they are the subject of this review.

## PERTUSSIS TOXIN

Pertussis toxin (PTX) is the most complex soluble bacterial exo-protein known to date. It is composed of five different subunits, named S1 through S5, according to their decreasing molecular weights. The subunits are arranged in a hexameric structure with the following stoechiometry : 1S1:1S2:1S3:2S4:1S5 (Tamura *et al.*, 1982). PTX is a member of the A-$B_5$ class of toxins, in which the A moiety expresses enzymatic activity, whereas the B moiety is responsible for the binding of the toxin to the target-cell receptors. The crystal structure of PTX has revealed that the B oligomer, composed of subunits S2 through S5, forms a triangular base on top of which the A protomer, composed of the S1 subunit, is anchored via its C-terminal end that extends into the pore of a central barrel formed by the B oligomer subunits (Stein *et al.*, 1994a). The toxin is actively secreted by *B. pertussis* (Weiss *et al.*, 1993) and plays an essential role in the pathogenesis of whooping cough (Pittman, 1984). As a major virulence factor, it is also a protective antigen and an important component of pertussis vaccines (Decker and Edwards, 1988).

### Biogenesis of PTX

The five PTX subunits are encoded by contiguous cistrons within a single polycistronic operon (Locht and Keith, 1986; Nicosia *et al.*, 1986), whose expression

is regulated by the BvgA/S system. The individual subunits are produced as pre-proteins containing cleavable signal peptides, and therefore cross the inner membrane of the bacterium most likely via a Sec-dependent pathway. Although the signal peptides show typical features of the substrates for leader peptidase I, their cleavage by the *Escherichia coli* leader peptidase I is very inefficient. Co-expression of the *B. pertussis lep* gene in *E. coli* substantially increases the maturation of PTX subunits, although the overall topology of the *B. pertussis* leader peptidase I is very similar to that of the *E. coli* enzyme (Smith *et al.*, 2000). However, not all *B. pertussis* pre-proteins require *B. pertussis*-specific leader peptidase I, as examplified by the *B. pertussis* porin protein, whose signal peptide is efficiently cleaved by the *E. coli* enzyme (Antoine and Locht, unpublished). The molecular details of this specificity remain to be investigated.

Once the individual subunits have reached the periplasmic space, and their signal peptides have been removed, the holotoxin is assembled prior to secretion through the outer membrane. A key step in toxin assembly is the correct disulphide bond formation. S1 contains 2 cysteines, whereas S4 and S5 each contain 4 cysteines and S2 and S3 each contain 6 cysteines (Locht and Keith, 1986; Nicosia *et al.*, 1986). All the cysteines are involved in intra-chain disulphide bonds (Stein *et al.*, 1994a). The disulphide formation is important for the toxin biogenesis, as the alteration of either cysteine in S1 prevents this subunit from assembling with the B oligomer (Antoine and Locht, 1990). The proper disulphide formation requires the action of the *B. pertussis* protein disulphide isomerases DsbA and DsbB. Mutations in either *dsbA* or *dsbB* result in decreased levels of S1 and S2, indicating that both enzymes are required (Stenson and Weiss, 2002). However, mutations in *dsbC*, coding for a third disulphide isomerase, have apparently no effect on the assembly of the toxin, although they impair its secretion, suggesting DsbC acts on a component that is specifically required for PTX secretion.

Full assembly of the holotoxin is important for efficient secretion, and strains engineered to produce only S1 or only the B oligomer show a defect in secretion (Farizo *et al.*, 2000). However, in some constructs, fully functional B oligomer can be assembled and secreted in the absence of S1 (Antoine and Locht, 1990), indicating that the presence of S1 is not an absolute prerequisite for B oligomer secretion. In contrast, S1 alone cannot be secreted in the absence of the B oligomer, suggesting that the secretion determinants are located within the B oligomer, although the presence of S1 may certainly enhance the secretion efficiency. Certain mutations in the S1 subunit gene have a strong deleterious effect on secretion, in particular the area around Arg-57 (Craig-Mylius *et al.*, 2000), suggesting that this region of S1 plays a role in the secretion of PTX. In the absence of the B oligomer, S1 most likely partitions to the outer membrane, perhaps via its C-terminal, hydrophobic domain. This may be the site of assembly with the B oligomer prior to secretion through the outer membrane (Farizo *et al.*, 2002).

The molecular steps in holotoxin assembly are still poorly understood. Single subunits in mutant strains that do not produce the other subunits are rapidly degraded. Certain combinations of subunits appear to mutually stabilize each other (Burns *et al.*, 2004). For example, the stability of the S2 subunit is greatly enhanced by the presence of S4 and vice versa, which is consistent with the S2-S4 dimer formation revealed by the crystal structure of the holotoxin. It is also possible that subassemblies of the S2-S4 dimer with the S1 subunit are formed in the absence of S3 and S5. The S2-S4 dimer has not been found to be secreted in *B. pertussis*, whereas the addition of S1 to this dimer can result in some secretion.

The transport of PTX through the outer membrane of *B. pertussis* relies on a type IV secretion system composed of nine different proteins, named PtlA through PtlI (Weiss *et al.*, 1993; Farizo *et al.*, 1996). These proteins are homologous to those of type IV secretion systems from other bacteria, including *Agrobacterium tumefaciens*, *Bartonella tribocorum*, *Brucella suis*, *Helicobacter pylori*, *Legionella pneumophila*, and *Rickettsia prowasekii* (Baron *et al.*, 2002). In all cases, these systems mediate secretion of proteins or protein-DNA complexes, which most often constitute virulence factors. The *ptl* genes lie directly downstream of the five structural PTX genes (Weiss *et al.*, 1993), and their expression depends on the *ptx* promoter (Kotob *et al.*, 1995). The *ptl* genes form together with the *ptx* genes most likely a single polycistronic operon (Baker *et al.*, 1995; Antoine *et al.*, 1996). Nevertheless, the Ptl proteins appear to be produced at lower levels than the PTX subunits, as investigated by translational fusions of the *phoA* gene with various *ptx* and *ptl* genes (Cheung *et al.*, 2004). The production of certain Ptl proteins appears to be a limiting step in the secretion of PTX. During exponential growth, the bacteria have been estimated to secrete three toxin molecules/min/bacterial cell (Rambow-Larsen and Weiss, 2004), and subunits have been found to accumulate within the periplasmic space, both as individual subunits and assembled into holotoxin. Subunit accumulation occurs throughout exponential growth even though the levels of certain Ptl proteins increase between 30 to 1,000 molecules per cell. Thus, secretion

rather than toxin production or assembly may be rate limiting. Curiously, in the absence of the Ptl proteins, some subunit combinations, such as the S2-S4 dimer in the presence of S1, can be secreted (Burns *et al.*, 2004), although the secretion of the holotoxin strongly depends on the presence of the Ptl proteins. Ptl-independent secretion has also been observed for strains producing only the S1 subunit without any of the B oligomer subunits (Farizo *et al.*, 2000).

The Ptl proteins are thought to form a complex spanning both the inner and the outer membranes (Johnson and Burns, 1994), and all of them (at least PtlA–H) appear to be needed for the toxin secretion, as investigated by mutations in each individual *ptl* gene (Craig-Mylius and Weiss, 1999). Some of the mutations introduced in *trans* into wild-type *ptl*$^+$ strains resulted in a dominant negative secretion phenotype, confirming that at least PtlC to PtlH are part of a multimeric complex. The role of certain individual Ptl proteins in the secretion of PTX has been investigated. A key step in PTX secretion is obviously its ability to cross the peptidoglycan layer, and PtlE has been shown to express peptidogycanase activity (Rambow-Larsen and Weiss, 2002). This 276-residue long protein contains active site similarities with other glycohydrolases, and alanine substitutions at this site have been shown to strongly diminish the peptidoglycanase activity of recombinant PtlE. The same substitutions in natural PtlE also abolish secretion of PTX by *B. pertussis*, strongly suggesting that PtlE is the peptidoglycanase required for the toxin to cross the peptidoglycan layer.

PTX secretion also most likely requires energy. Two of the Ptl proteins, PtlC and PtlH, contain putative ATP-binding sites and might thus provide the energy necessary for toxin secretion. Both proteins have been shown to be necessary for secretion (Kotob and Burns, 1997; Cook *et al.*, 1999), and in both cases, the putative ATP-binding sites are essential for function. For both, a dominant negative phenotype is observed when mutant alleles are co-expressed with the wild type allele, suggesting that both function as multimers or are part of the secretion complex. However, the reason of this redundancy is not known, and it is not clear what the relative contribution of PtlC and PtlH is in energy supply and toxin secretion.

The functional role of the other Ptl proteins is not yet known, but some of them might perhaps be inferred by analogy to the *A. tumefaciens* VirB system. PtlF shows features of outer membrane proteins, but it may also be associated with the inner membrane, like its VirB9 homologue. In non-reducing conditions, PtlF and PtlI migrate as a complex during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that PtlI binds to PtlF by disulphide bond formation (Farizo *et al.*, 1996). The proper disulphide bond formation between PtlI and PtlF might depend on DsbC, as mutations in the *dsbC* gene affect secretion without affecting toxin assembly (Stenson and Weiss, 2002).

## Structure-function relationship

The molecular events of PTX action can be divided into four major steps: (i) target-cell receptor binding, (ii) intracellular trafficking, (iii) membrane translocation of the enzymatically active S1 subunit, and (iv) expression of ADP-ribosytransferase activity of S1.

Binding of the toxin to its target-cell receptors occurs via the B oligomer. Virtually all eukaryotic cells display PTX receptors at their surface, although the nature of these receptors may vary from cell type to cell type. The toxin receptors identified so far are all glyco-conjugates, generally sialoglycoproteins (Armstrong *et al.*, 1988), although certain peptides can mimic the glycosidic moiety of the receptors (Bogdan *et al.*, 2003), suggesting that glycosylation may not necessarily be an absolute requirement. On the other hand, other glyco-conjugates, such as the glycolipid $G_{D1a}$, may also serve as PTX receptors (Hausman and Burns, 1993). Receptor proteins of sizes between 43 kDa and 200 kDa have been identified (Rogers *et al.*, 1990; El Baya *et al.*, 1995), depending on the cell type. Various treatments of the sugar moieties of the receptor glycoproteins have indicated the importance of the terminal sialyl groups in PTX binding (Brennan *et al.*, 1988).

The broad range specificity of PTX receptor binding may explain the wide range of biological activities expressed by the toxin and may be the consequence of its particularly complex quaternary structure. The crystal structure of the oligosaccharide-bound form of PTX revealed the presence of at least two distinct carbohydrate-binding sites (Stein *et al.*, 1994b). One of them involves the S2 subunit and one involves S3. The two subunits display approximately 70% amino acid sequence identity (Locht and Keith, 1986; Nicosia *et al.*, 1986). Each one of these subunits dimerizes with S4, and the receptor-binding units are probably constituted by the S2/S4 and the S3/S4 dimers. Although both dimers are able to bind to sialo-carbohydrates in essentially the same manner, they nevertheless differ in their receptor specificity. For example, dimer S2/S4 is able to bind to haptoglobin, whereas dimer S3/S4 binds to glycoproteins at the surface of Chinese hamster ovary cells (Lobet *et al.*, 1993). The C-terminal half of S2 and of S3 adopt a fold similar to that found in the B subunits of other carbohydrate-binding toxins, and the sialyl group of the oligosaccharide bound to PTX is within hydro-

gen-bonding distance of the polar and charged groups of Tyr-102, Ser-104, and Arg-125 in both S2 and S3 (Stein *et al.*, 1994b). Site-specific alterations of the residues in that region abolish receptor binding of PTX (Lobet *et al.*, 1993; Loosmore *et al.*, 1993). Interestingly, deletion of Asn-105 in S2 affects haptoglobin binding of the toxin, while keeping its ability to bind to CHO cells intact, whereas the deletion of Lys-105 in S3 has the inverse effect (Lobet *et al.*, 1993), indicating that these residues contribute to receptor-binding specificity. Other regions, in particular in the N-terminal domain of these subunits, may also be involved in receptor recognition (Saukkonen *et al.*, 1992).

Upon binding to its receptors, the toxin enters the target cells by receptor-mediated endocytosis. This does probably not require a conformational change of the toxin (Shigeta, 1994). Several lines of evidence suggest that the toxin follows the retrograde transport system involving the Golgi apparatus and possibly the endoplasmic reticulum (ER) (Xu and Barbieri, 1995; El Baya *et al.*, 1997). The addition of $NH_4Cl$ or chloroquine to the target cells inhibits PTX action, suggesting that a pH-sensitive step is involved in toxin trafficking. However, membrane translocation of PTX does not appear to depend on a pH gradient (Hausman and Bruns, 1992), suggesting that the translocation step does not occur in acidified endosomes. Much of the toxin may actually travel back to the cell surface (Xu and Barbieri, 1995). The compartment where toxin translocation occurs is not yet known.

The PTX domains involved in toxin translocation also have not yet conclusively been determined. Unlike diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, PTX does not contain a well-defined translocation domain in its B moiety. The fact that S1 is able to bind to phospholipid bilayers (Hausman and Burns, 1992) suggests that the S1 subunit may directly interact with the membrane and mediate its own membrane translocation. The B oligomer may thus perhaps not be essential for the membrane translocation step *per se*. This hypothesis is supported by results obtained from cell transfection experiments (Veithen *et al.*, 2000; Castro *et al.*, 2001) showing that when the S1 subunit gene is expressed in CHO cells with a signal-peptide coding sequence, the subunit is targeted to the ER. The matured S1 then travels back to the cytosol and expresses its enzymatic activity, which results in a toxic phenotype of the CHO cells in the absence of the B oligomer. Consistent with these data, Hazes and Read (1997) have proposed a model in which S1 dissociates from the B oligomer in the ER and translocates without further help from the B oligomer. This model is based on the crystal structure of the PTX-ATP complex (Hazes *et al.*, 1996). The negative charges of the triphosphate moiety of ATP displace the C-terminal, negatively charged residues of S1 from the B-oligomer pore, resulting in a destabilization of the S1-B oligomer interactions and ultimately the release of S1 from the B oligomer. Since, in addition to the cytosol, the ER is the only subcellular compartment where ATP can be found, Haze and Read (1997) suggest that the release of S1 from the B oligomer occurs in the ER. Furthermore, this compartment also contains protein disulphide isomerases that may help to reduce the intramolecular disulphide bond of S1, which is necessary for the expression of the enzymatic activity.

Cytosolic, reduced S1 catalyzes the transfer of the ADP-ribose moiety of NAD onto acceptor substrate proteins. The acceptor substrates are the α subunits of a class of signal-transducing G proteins (Katada and Ui, 1982). In particular, PTX ADP-ribosylates Cys-351 of Gi/o, located near the C-terminus of their α subunits. ADP-ribosylation of this residue results in uncoupling of the G protein from its cognate receptor and disruption of signal transduction. Although Cys-351 of Giα is the natural acceptor substrate of PTX, the toxin can also hydrolyze NAD in a reaction referred to as the NAD-glycohydrolase reaction. This amounts to the transfer of ADP-ribose to a water molecule.

The enzymatic mechanism of PTX has been extensively studied (Locht and Antoine, 1995). Catalytic residues have been identified, as well as the residues or regions involved in NAD binding and in acceptor substrate binding (Locht *et al.*, 2000). The C-proximal portion of S1 is involved in the interaction with Gα (Locht *et al.*, 1990; Cortina *et al.*, 1991), and the region around Trp-26 and Arg-9 appears to be involved in NAD binding (Cortina and Barbieri, 1989). The two catalytic residues that have been identified by the role they play in the velocity of the reaction are His-35 (Antoine and Locht, 1994) and Glu-129 (Antoine *et al.*, 1993). Both residues are within hydrogen-bonding distance from each other (Stein *et al.*, 1994a), and Glu-129 is strictly conserved among all ADP-ribosyltransferases investigated so far (Pallen *et al.*, 2001). We have proposed that Glu-129 weakens the N-glycosidic bond linking ADP-ribose to the nicotinamide ring by stabilizing or promoting the formation of an oxacarbenium-like intermediate. This may occur through ionization of the nicotonamide ribose diol, which would result in intramolecular electrostatic stabilization of the intermediate. The existence of an oxacarbenium-like ribose ring at the transition state has been demonstrated by isotope effect characterization of the transition state in the PTX-catalyzed NAD-glycohydrolase reaction (Scheuring and Schramm, 1997). Recently, analogs of NAD that resemble their oxacarbenium ion transition

states have been shown to inhibit the NAD-glycohydrolase reaction catalyzed by PTX (Zhou *et al.*, 2004). His-35 probably increases the nucleophilicity of the acceptor substrate to enhance its attack of the weakened N-glycosidic bond, ultimately resulting in the transfer of ADP-ribose onto the acceptor substrate, probably by an SN2-type mechanism. $D_2O$ kinetic isotope effects are consistent with the activation of the acceptor cysteine through deprotonation prior to the nucleophilic attack (Scheuring and Schramm, 1997b). His-35 is less well conserved among the ADP-ribosyltransferases, as the acceptor substrates of these enzymes vary.

## Role of PTX in pathogenesis and protection against whooping cough

PTX expresses a wide variety of different biological activities. They include the induction of morphological changes of target cells, of exocrine secretion, of the stimulation of lipolysis, the activation of pancreatic islet cells, histamine sensitization, the induction of lymphocytosis, and many others. Therefore, PTX was originally given different names, such as islet-activating protein, histamine sensitization factor, and lymphocytosis promoting factor. It was only in the early 1980s through the work of Munoz on very pure, crystallin PTX that all these different activities were related to a single molecule, named *pertussigen* by Munoz (Munoz, 1985), and now known as PTX. Most of the biological activities of PTX *rely* on its ADP-ribosyltransferase activity. However, a few, such as hemagglutination and mitogenicity, are independent of the ADP-transfer reactions, but depend on certain target-cell receptor-binding properties of PTX (Lobet *et al.*, 1993; Loosemore *et al.*, 1993).

Some of the biological activities of PTX are hallmarks of whooping cough, which has led to the proposal that pertussis is essentially a toxin-mediated disease, similar to tetanus and diphtheria (Pittman, 1984). However, through genomic studies, it has now become clear that the pathogenic mechanisms of pertussis are far more complicated than initially assumed, and that many additional virulence factors contribute to the pathogenesis of the disease (Locht *et al.*, 2001).

Nevertheless, PTX plays a key role in the pathogenesis. A PTX-deficient mutant strain is severely impaired in its ability to cause lethal infections in infant mouse models and is cleared more rapidly that an isogenic PTX-producing strain, although in the initial phases, the colonization profiles do not differ significantly between the two strains (Goodwin and Weiss, 1990). In adult mice, PTX-deficient strains induce much less lung pathology than PTX-producing strains, although both types of strains are able to colonize adult mouse lungs equally well.

Interestingly, intranasal infection with PTX-deficient strains induces a stronger antibody response to other *B. pertussis* antigens, such as filamentous hemagglutinin, than infection with PTX-producing strains, indicating that PTX has immunosuppressive functions *in vivo*. These functions depend on the ADP-ribosyltransferase activity. One reason for the immunosuppressive function of PTX may lie in its ability to reduce HLA-DR surface expression in infected monocytes (Shumilla *et al.*, 2004). In addition, PTX has been shown to inhibit the ability of monocytes to internalize bacteria, suggesting that the inhibition of monocyte phagocytosis by PTX may represent an immune evasion mechanism of *B. pertussis* (Schaeffer and Weiss, 2001). However, these observations are in contrast to previously described adjuvant activities of PTX. These contrasting observations may be due to differences in the route and time of administration of PTX, relative to the target antigen, as well as the amount of PTX used in the different studies.

Such apparent paradoxical effects have also been observed in experimental autoimmune disease models, where PTX has been found to have enhancing and inhibitory effects, depending on its time of administration. Also, the administration of high doses of PTX led to strongly increased cytokine responses, enhanced adaptive Th1 immunity, and disease, whereas low doses of PTX suppressed experimental autoimmune uveoretinitis (Agarwal *et al.*, 2002). The inhibitory effect appeared to be due to the ADP-ribosyltransferase activity, whereas disease induction and enhancement of delayed-type hypersensitivity was independent of enzyme activity of PTX and largely due to the B oligomer of the toxin (Su *et al.*, 2003). The immune enhancing effects of PTX probably result from PTX-mediated stimulation of dendritic cells, as injection of dendritic cells stimulated *in vitro* with PTX is able to substitute for PTX injection to promote experimental autoimmune uveitis (Hou *et al.*, 2003), suggesting that PTX promotes the maturation of dendritic cells. PTX-mediated maturation of dendritic cells has been shown, but, strangely, this activity appeared to depend on the ADP-ribosyltransferase of PTX and could even be mimicked by cAMP analogs, showing that the PTX-mediated maturation of dendritic cells is cAMP-mediated (Bagley *et al.*, 2002). The activation of antigen-presenting cells by PTX has been shown to result in antigen-specific clonal expansion of both $CD4^+$ and $CD8^+$ T cells, as well as in substantially elevated levels of IL-5 and IFN-γ (Shive *et al.*, 2000). Elegant studies using Gi2α knockout mice have further suggested that Gi2α protein-mediated signaling is involved at least in the induction of Th1 type

responses to antigens co-administered with PTX (He *et al.*, 2000). These observations are at odds with the above described ADP-ribosyltransfer-independent adjuvant activities of PTX, and in contrast with a report describing that maturation of myeloid dendritic cells and the induction of IL-12 and TNF-α by PTX does not require its enzymatic activity (Tonon *et al.*, 2002). The reasons for this controversy are currently not known, although it should be noted that in the latter studies very high concentrations of PTX were used.

In addition to its action on immune cells, PTX action on epithelial cells may also be critical for the regulation of inflammatory reactions in the respiratory tract of *B. pertussis*-infected subjects. When human respiratory epithelial cells are infected with PTX-deficient mutants, higher levels of ICAM-1 mRNA are induced than when they are infected with PTX-producing strains, and purified PTX suppresses the *B. pertussis*-induced up-regulation of ICAM-1 (Ishibashi and Nishiwawa, 2002). These observations suggest that PTX may decrease immune responses by down-regulating surface expression of adhesion molecules on epithelial cells, thereby limiting immune cell recruitment at the site of infection.

Given the important role of PTX in the pathogenesis of pertussis and because of its immunomodulatory properties, this molecule can be used for several potential applications. An interesting application may result from the ability of the toxin to inhibit tumor cell motility both *in vitro* and *in vivo*. It has therefore been proposed as a therapeutic agent against certain forms of cancer, such as bladder cancer, and initial phase I studies in humans have shown that intravesical instillation of PTX is safe and well tolerated in dosages at least up to 72 mg (Otto *et al.*, 1999).

Probably the most widely used application of PTX is its inclusion as a protective antigen in vaccines against whooping cough. PTX has long been recognized as a protective antigen in animal models. In fact, vaccination with PTX alone is sufficient to fully protect mice against intracerebral challenge with virulent *B. pertussis* (Sato *et al.*, 1991). Therefore, PTX has been included in all the acellular vaccines currently available, although these vaccines vary substantially with respect to the inclusion of other antigens, such as filamentous hemagglutinin, pertactin, and the fimbriae (Decker and Edwards, 2000). It has even been suggested that detoxified PTX can be used alone without any of the other *B. pertussis* antigens to develop efficacious vaccines against whooping cough (Taranger *et al.*, 2001). On the other hand, it may also be possible to develop efficacious vaccines in the absence of PTX, as infection with a PTX-deficient strain is able to fully protect mice against subsequent challenge with a virulent strain (Mielcarek *et al.*, 1998).

## ADENYLATE CYCLASE TOXIN

The adenylate cyclase (ACT, AC-Hly, or CyaA) is the only known enzyme toxin capable of directly penetrating target cells across the cytoplasmic membrane and of reaching the cytosol without the need for endocytosis (Gordon *et al.*, 1988; Bellalou *et al.*, 1990; Schlecht *et al.*, 2004). This mode of entry into cells by self-supported membrane translocation is accomplished by a single CyaA polypeptide of 1706 residues (Figure 16.1). The toxin molecule consists of an N-terminal ~400 residue-long adenylate cyclase enzyme (AC) domain that is linked to a characteristic RTX hemolysin (Hly) moiety of ~1300 residues (Glaser *et al.*, 1988a). The Hly portion serves both for the secretion of CyaA out of the bacteria (Glaser *et al.*, 1988b) and for toxin binding to the $\alpha_M\beta_2$ integrin receptor (CD11b/CD18, CR3, or Mac-1) of myeloid phagocytic cells (Guermonprez *et al.*, 2001; El-Azami-El-Idrissi *et al.*, 2003) and delivery (injection) into the target cells (Bellalou *et al.*, 1990).

The toxin was discovered in 1975 by Hewlett *et al.* (1976) as an extracytoplasmic AC enzyme activity present in *B. pertussis* culture media. Wolff *et al.* (1980) found in 1980 that the activity of the AC enzyme was enhanced by several orders of magnitude following interaction with eukaryotic calmodulin. Confer and Eaton (1982) then showed that CyaA can penetrate phagocytic cells and provokes their impotence by elevating intracellular levels of cAMP, a key cellular "second messenger" signaling molecule. Finally, Weiss *et al.* (1986) demonstrated in 1986 that the toxin plays a key role in *Bordetella* virulence, by showing that *cyaA::Tn5* mutants unable to produce CyaA exhibit a substantially higher $LD_{50}$ in the infant mice model of intranasal *B. pertussis* infection than wild-type strains. The toxin was subsequently found to play a particularly important role in the early stages of *Bordetella* colonization of the host respiratory tract (Goodwin and Weiss, 1990; Khelef *et al.*, 1992) and, in contrast to PTX, CyaA is produced also by *B. parapertussis* and *B. bronchiseptica* (Parkhill *et al.*, 2003).

### Biogenesis of CyaA

CyaA belongs to the steadily growing family of RTX (*R*epeat-in-*T*o*X*in) proteins of Gram-negative bacteria that are secreted through the type I pathway and harbor the calcium-binding aspartate and glycine-rich G-G-X-G-X-D repeats (Welch, 2001). The toxin is encoded

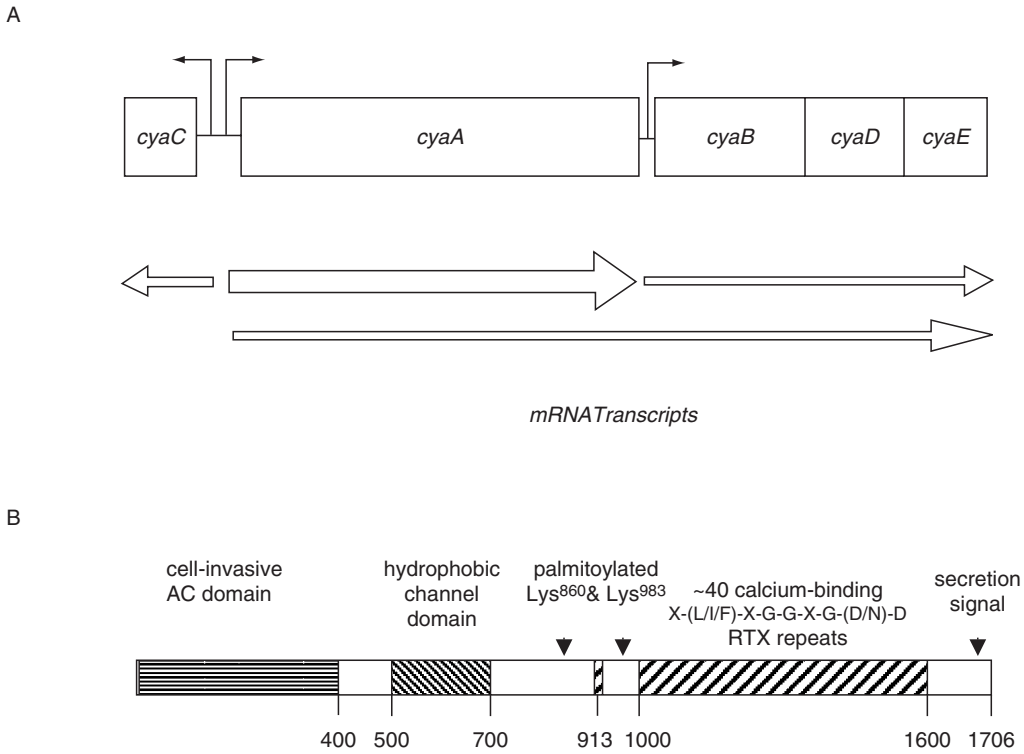



**FIGURE 16.1** (A) Organization of the *cya* locus of *Bordetella pertussis*. The *cyaABDE* genes (Glaser *et al.*, 1988a) form an operon transcribed from a BvgA/S-regulated promoter located 115 bp upstream from the translational start codon of the protoxin (proCyaA) gene *cyaA*. The transcript is partially terminated 3′ to the *cyaA*, with some read-through into the downstream genes resulting in full-length *cyaABDE* transcripts. The *cyaBDE* genes coding for the components of a the type I secretion apparatus are constitutively transcribed at a lower level from a BvgA/S-independent promoter located in the intergenic *cyaA-cyaB* region (Laoide and Ullmann, 1990). The *cyaC* gene is transcribed in the opposite direction and encodes the 22-kDa CyaC protein responsible for the posttranslational fatty-acyl modification of proCyaA (Barry *et al.*, 1991).
(B) Organization of the CyaA toxin molecule. CyaA consists of an N-terminal ~400 residue-long, cell-invasive, and CaM-activated AC domain (Wolff *et al.*, 1980; Confer and Eaton, 1982; Glaser *et al.*, 1989) and of a ~1300 residue-long RTX hemolysin/cytolysin (Hly) moiety (Glaser *et al.*, 1988a; Sakamoto *et al.*, 1992). The Hly portion contains (i) between residues 500 to 700 a hydrophobic channel-forming domain (Benz*et al.*, 1994); (ii) between residues 800 to 1,000 a domain where the activation by posttranslational palmitoylation of $Lys^{860}$ and $Lys^{983}$ takes place (Hackett *et al.* 1994, 1995); (iii) between residues 1,000 to 1,600 an RTX domain containing the characteristic repeats formed by X-(L/I/F)-X-G-G-X-G-(N/D)-D nonapeptides into which up to 40 calcium atoms bind with low-affinity (Rose *et al.*, 1995) and a C-terminal secretion signal (Sebo and Ladant, 1993).

by the *cya* locus (Figure 16.1A), and its production is under control of the BvgA/S system (Scarlato *et al.*, 1991). Of the five *cya* genes in the locus, the *cyaABDE* genes are grouped within an operon (Glaser *et al.*, 1988a; Laoide and Ullmann, 1990), while the *cyaC* gene is transcribed in an opposite direction. It encodes the 22-kDa CyaC protein, an acyltransferase that catalyzes the posttranslational fatty-acyl modification converting proCyaA to the mature and biologically active CyaA (Barry *et al.*, 1991). The *cyaABDE* operon starts by the structural *cyaA* gene, followed by three genes encoding the inner membrane ATP-ase (CyaB), the membrane fusion protein (CyaD), and the outer membrane channel component (CyaE) of a typical type I protein secretion system (Glaser *et al.*, 1988b). The toxin is likely to be secreted by a mechanism identical to that used for secretion of the RTX α-hemolysin (HlyA) of *E. coli* through the HlyBD/TolC apparatus (Koronakis *et al.*, 2004). By analogy, CyaB would operate as an ABC family transporter that recognizes the secretion signal located within the last 74 residues of CyaA (Sebo and Ladant, 1993) and drives secretion of an unfolded CyaA polypeptide through a tightly packed trimeric CyaBDE "channel-tunnel" conduit, directly from bacterial cytoplasm into the external medium, without passage of CyaA through the periplasmic compartment (Koronakis *et al.*, 2004). In many *Bordetella* isolates, the protein remains attached to the bacterial surface following secretion, due to an interaction with the filamentous hemaglutinin (Zaretzky *et al.*, 2002). However, most of the cell-attached CyaA appears to be aggregated and unable to act as a "contact weapon"

since only the newly secreted CyaA is capable of penetrating target cells and raising intracellular cAMP levels (Gray *et al.*, 2004).

## The RTX hemolysin moiety of CyaA

The RTX hemolysin (Hly) moiety of CyaA (~1300 carboxy-proximal residues) is functionally independent and is itself capable of forming small cation-selective membrane channels of a diameter between 0.6 to 0.8 nm (Sakamoto *et al.*, 1992; Benz *et al.*, 1994). These channels can permeabilize target cell membranes and provoke colloid-osmotic lysis of erythrocytes, accounting for the hemolytic halo of *Bordetella* colonies on blood agar plates (Weiss *et al.*, 1984). However, compared to typical hemolysins of the RTX family, the specific hemolytic activity of CyaA is low (Bellalou *et al.*, 1990; Ehrmann *et al.*, 1991), suggesting that the main role of the Hly moiety is not hemolysis *per se*, but rather supporting delivery of the AC domain into target cells (Rogel *et al.*, 1991; Rogel and Hanski, 1992).

The RTX Hly of CyaA consists itself of several functional subdomains (Iwaki *et al.*, 1995; Rose *et al.*, 1995). It contains (i) a hydrophobic channel-forming domain comprising residues 500 to 700 (Benz *et al.*, 1994); (ii) an activation domain between residues 800 and 1000, where the posttranslational palmitoylation of CyaA occurs (Hackett *et al.*, 1994; Hackett *et al.*, 1995); and (iii) a typical calcium-binding RTX domain, harboring the more-or-less conserved nonapeptide repeats of a consensus sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D, which form numerous (aproximedtely 40) calcium-binding sites (Rose *et al.*, 1995; Rhodes *et al.*, 2001). Cooperation and structural integrity of all domains of the Hly moiety appear to be critical for membrane insertion and translocation of the AC domain into the target cell cytosol (Bellalou *et al.*, 1990; Iwaki *et al.*, 1995). The main segment of CyaA required for binding to the CD11b/CD18 integrin receptor has been recently located in the glycine-rich repeat region between residues 1166 to 1281 of CyaA (El-Azami-El-Idrissi *et al.*, 2003).

## The cell-invasive adenylate cyclase enzyme domain of CyaA

Following penetration into target cells, the AC domain binds intracellular calmodulin (CaM) in a 1:1 stoichiometry, and its enzymatic activity is increased by >1000-fold, reaching the extremely high catalytic power of $k_{cat}$ ~2000 $s^{-1}$. This appears to be about three orders of magnitude higher than the activity of endogenous cellular adenylate cyclases (Ladant, 1988; Glaser *et al.*, 1989). Upon limited proteolysis of the AC-CaM complex with trypsin, two functional subdomains of the catalytic AC domain could be defined as T25 and T18 fragments that are still able to assemble with CaM into a fully active ternary complex (Ladant, 1988). By using residue modification and mutagenesis, the AC catalytic site was located within the T25 subdomain (residues 1–224), while the main CaM-binding domain was located on the T18 (residues 225–399) fragment (Glaser *et al.*, 1989; Ladant *et al.*, 1989). Amino acid substitutions of $Trp^{242}$ have yielded AC enzymes with a full catalytic activity and up to 1000-fold reduced affinity for CaM (Glaser *et al.*, 1989; Ladant *et al.*, 1989). The hydrophobic side of the α-helical region situated around $Trp^{242}$ is the major site of the AC domain interaction with CaM, supporting the conclusion that the secondary rather than the primary structure of the binding site is important for interaction with CaM (Bouhss *et al.*, 1993). However, AC appears to make contact with CaM at multiple sites, as a 71 residue-long peptide, comprising residues 196 to 267 of CyaA and overlapping with the carboxyl terminus of T25 and the amino terminus of T18, is needed to recover 90% of the binding energy of the AC-CaM complex (Bouhss *et al.*, 1993).

The *Bordetella* AC domain was found to share three short homologous protein segments (I to III) with edema factor (EF) of *Bacillus anthracis*, the other known CaM-dependent bacterial AC enzyme (Glaser *et al.*, 1991). This similarity allowed investigators to pinpoint several residues that are critical for the catalytic activity of AC. $Lys^{58}$ and $Lys^{65}$ within segment I (residues 54–77) were found to be part of the AC catalytic site, and their replacements result in an important decrease, or total loss, of enzyme activity (Glaser *et al.*, 1989). In addition, $His^{63}$ in segment I has been identified as the general acid/base catalyst in a predicted charge-relay system proposed to be involved in the reaction mechanism of adenylyl cyclization (Munier *et al.*, 1992). In segment II (residues 184–196), $Asp^{188}$ and $Asp^{190}$ appear to be required for binding of the $Mg^{2+}$-ATP complex (Glaser *et al.*, 1991). Finally, substitutions of $His^{298}$ and $Glu^{301}$ in segment III (residues 294–314) also affect the nucleotide binding properties of AC, albeit to a lesser degree (Glaser *et al.*, 1991). The original conclusion, that $His^{298}$ and $Glu^{301}$ are involved in the mechanism of AC activation by CaM, may need to be revised, given that the corresponding segment of *B. anthracis* EF (residues $Asn^{583}$, $Glu^{588}$, and $Asp^{590}$) clearly appears to be involved in catalysis (Drum *et al.*, 2002). In general, however, the recent structural work on EF suggests that mutagenesis of AC yielded a fairly accurate identification of the essential catalytic residues of CyaA, with $His^{63}$ of CyaA corresponding well to $His^{351}$ that serves as a general catalytic base in EF (Drum *et al.*, 2002). The

studies on EF revealed a novel reaction mechanism for the CaM-activated bacterial AC, which differs from the two-metal-ion catalytic mechanism of mammalian enzymes. However, it remains to be determined whether AC of CyaA uses a similar reaction mechanism as EF, since the AC and EF enzymes exhibit substantial differences in CaM binding and activation, and the entire helical domain of EF is missing in the AC enzyme (Shen *et al.*, 2002). The ~10,000-fold activation factor of AC upon CaM binding is higher than that of EF, and AC exhibits an about 100-fold higher affinity for CaM than EF. The $K_d$ of AC for CaM ranges between ~20 nM and ~2 nM at physiological concentrations of intracellular $Ca^{2+}$ and CaM (50 nM to 10 fM) and is decreased to about ~0.2 nM at 0.1 mM free $Ca^{2+}$ concentrations (Ladant *et al.*, 1992; Shen *et al.*, 2002).

## Role of $Ca^{2+}$ ions in toxin activity of CyaA

Calcium ions are required for CyaA binding to the $\alpha_M\beta_2$ integrin receptor (CD11b/CD18) on myeloid cells (Guermonprez *et al.*, 2001), and the toxin activity of CyaA strictly requires exposure of the protein to physiological (>0.3 mM) concentrations of free calcium ions (Hanski and Farfel, 1985; Hewlett *et al.*, 1991). CyaA harbors numerous (about 40) calcium binding sites, and already a seemingly "irreversible" loading of a small number of $Ca^{2+}$ binding sites (about 5) allows the toxin to interact with membranes and to express hemolytic activity (Rogel and Hanski, 1992; Rose *et al.*, 1995). The precise location of these high-affinity binding sites within the Hly moiety of CyaA remains unknown, while the low-affinity ($K_d$ >0.3 mM) $Ca^{2+}$ binding sites (~35-40) have been located within the 700 C-terminal residues of CyaA (Rose *et al.*, 1995; Rhodes *et al.*, 2001). By analogy with the structure of the RTX moiety of the alkaline protease of *Pseudomonas* (Baumann *et al.*, 1993), the calcium atoms are expected to bind to CyaA within the turns connecting adjacent anti-parallel beta strands formed by the X-(L/I/F)-X-G-G-X-G-(N/D)-D nonapeptides of the RTX domain. Calcium ions bind to CyaA in an extremely cooperative manner, and the channel-forming activity of CyaA is enhanced by ~50-fold upon a minimal (15%) increase of the free $Ca^{2+}$ concentration from 0.6 to only 0.8 mM (Knapp *et al.*, 2003). This suggests that following secretion from the calcium-poor bacterial cytoplasm into the body fluids containing millimolar concentrations of calcium, the $Ca^{2+}$ ions cooperatively bind to CyaA at numerous sites in parallel. This would account for the massive structural differences between the calcium-free and calcium-loaded CyaA molecules that have been observed earlier by negative staining electron microscopy (Hewlett *et al.*, 1991). Such "toggle-like" switching of the CyaA protein from an "off" to the "on" conformation would then support toxin penetration into target cell membranes, formation of CyaA channels, and delivery of the AC domain into cell cytosol.

## Posttranslational activation of CyaA by fatty-acylation

Similar to the other cytolytic RTX toxins, the capacity of CyaA to bind to and penetrate cellular membranes depends on activation by posttranslational modifications (Rogel *et al.*, 1989). For CyaA this is mediated by the co-expressed accessory protein CyaC (Barry *et al.*, 1991; Sebo *et al.*, 1991), which catalyzes covalent palmitoylation of the ε-amino groups of $Lys^{860}$ and $Lys^{983}$ in the conserved RTX acylation sites of CyaA (Hackett *et al.*, 1994; Hackett *et al.*, 1995).

Acylation of CyaA is required for the high-affinity ("irreversible") binding of CyaA to the CD11b/CD18 integrin receptor (El-Azami-El-Idrissi *et al.*, 2003). Interestingly, acylation also determines the capacity of CyaA to induce protective immunity against *Bordetella* infection (Betsou *et al.*, 1993), suggesting that acylation alters the overall conformation of CyaA. Native CyaA extracted from the *B. pertussis* strain 338 is acylated at the single $Lys^{983}$ residue (Hackett *et al.*, 1994), and single acylation of only $Lys^{983}$ appears to be necessary and sufficient for toxin activity on the so far examined target cell types. Furthermore, acylation of $Lys^{983}$ is sufficient for CyaA penetration of membranes of cells lacking CD11b/CD18, such as sheep erythrocytes (Basar *et al.*, 2001). However, recombinant CyaA produced in *E. coli*, or CyaA overproduced by a recombinant *B. pertussis* strain 18323/pBN, have both been found to be acylated on both conserved lysine residues, raising a question about the biological significance of the second acylation of CyaA (Havlicek *et al.*, 2001). Recently, we found that acylation of either $Lys^{860}$ or $Lys^{983}$ individually confers the full capacity of CyaA to tightly associate with CD11b/CD18-expressing cells. Moreover, CyaA acylated only on $Lys^{860}$ still exhibited a fairly high cytotoxic activity towards murine monocytic cells expressing $CD11b^+$ (Masin *et al.*, unpublished). This suggests that acylation of the two lysine residues is partially redundant in function, and it remains unclear whether acylation of both lysines is required for toxin activity on some as yet unknown targets.

Unexpectedly, acylation of CyaA is not essential for toxin interaction with lipid membranes, since the non-acylated proCyaA is even more active in penetrating and disrupting liposomes than acylated CyaA (Masin

*et al.*, 2004). However, the acyl residues attached to CyaA appear to play a role in modulating the protein-protein interactions that underlie toxin oligomerization and formation of the CyaA channels. This is suggested by the observation that acylation of recombinant CyaA by the C16:1 palmitoyl residues causes a highly selective decrease of the channel-forming (oligomerization) capacity of recombinant CyaA, as compared to the *B. pertussis* CyaA acylated by C16:0 palmitoyl chains, while both proteins exhibit an equal capacity to penetrate cellular membranes and to translocate the AC domain into cells (Benz *et al.*, 1994; Hackett *et al.*, 1995; Havlicek *et al.*, 2001). A structural role of acylation in promoting formation of toxin channels is also consistent with the observation that the high cation selectivity of the CyaA channel strongly depends on neutralization of the positive charge of $Lys^{983}$ by fatty-acylation (Masin *et al.*, unpublished). This indicates that acylated $Lys^{983}$ may be located at the mouth or within the CyaA channel.

## Membrane interaction and penetration of CyaA into cells

The CD11b/CD18 integrin is the high-affinity receptor for CyaA on myeloid phagocytic cells (Guermonprez *et al.*, 2001), which appear to be the physiologically most relevant cellular targets of the toxin (Harvill *et al.*, 1999). However, CyaA can also penetrate and intoxicate a variety of other cell types lacking CD11b/CD18, such as mammalian erythrocytes (Hanski, 1989; Bellalou *et al.*, 1990). Several reports have shown that CyaA is unique among the enzymatically active toxins in its capacity to cross directly the cytoplasmic membrane of cells and to reach their cytosol without the need for endocytosis. Intoxication of cells occurs instantaneously upon exposure to CyaA (Gentile *et al.*, 1988), and inhibitors of the known endocytosis mechanisms block only the CD18-dependent trafficking of CyaA for endosomal degradation and not the accumulation of intracellular cAMP (Gordon *et al.*, 1988; Guermonprez *et al.*, 1999), or the AC domain-mediated delivery of foreign epitopes into cell cytosol (Schlecht *et al.*, 2004).

Translocation, but not the mere insertion of CyaA into the cytoplasmic membrane of cells, appears to be driven by membrane potential (Otero *et al.*, 1995). However, the path of translocation of the ~40 kDa AC domain of CyaA across the lipid bilayer remains poorly defined. The AC does not seem to enter cells through the CyaA hemolysin channel, which has a diameter of only 0.6 to 0.8 nm and is thus too small for the passage of even a fully unfolded AC (Benz *et al.*, 1994). Formation of hemolytic CyaA channels is not a prerequisite for AC domain translocation into cells, as clearly shown by comparisons of CyaA activities on human and sheep erythrocytes and/or by comparison of activities between recombinant and native CyaA (Betsou *et al.*, 1993; Hackett *et al.*, 1995; Gray *et al.*, 1998). Moreover, translocation of the AC domain can be dissociated from the formation of the cation-selective CyaA channels by amino acid substitutions in the predicted membrane segments of CyaA (Osickova *et al.*, 1999). Mutant forms of recombinant CyaA have recently been generated, which exhibit an about twenty-fold lower channel-forming activity than native CyaA, while both forms of the protein are equally capable of translocating the AC domain into the cytososol of $CD11b^+$ monocytic cells (Basler, Masin, and Sebo, unpublished). Altogether, the available data allow us to propose a model of CyaA action in which two different and more-or-less mutually exclusive toxin conformers co-exist and insert into the target-cell membrane in parallel (Figure 16.2). One of these forms gives rise to a translocation precursor delivering the AC domain across the cytoplasmic membrane, while the other form inserts as a channel precursor and oligomerizes within the membrane to form the CyaA channel. However, the possibility still remains that CyaA interacts with the cellular membrane as a very stable dimer, since this would be indistinguishable from a monomer in the toxin activity versus concentration plots. The capacity of CyaA to form functional oligomers is also suggested by the rather efficient functional complementation of pairs of individually inactive toxin molecules carrying non-overlapping deletions (Iwaki *et al.*, 1995; Bejerano *et al.*, 1999).

## Physiological consequences of CyaA interactions with target cells

The role of CyaA in the interaction of *Bordetella* with the cells of the respiratory epithelia and in the modulation of the host immune response through induction of proinflammatory cytokine secretion remains poorly explored. Inflammation is observed in lungs of patients who died of whooping cough, and infection of human bronchial epithelial cells with *B. pertussis* upregulates mRNA expression of a number of genes encoding proinflammatory cytokines and induces IL-6 and IL-8 secretion (Belcher *et al.*, 2000). Recent work suggests that the CyaA activity may account for the induction of IL-6 in tracheal epithelia colonized by *B. pertussis* (Bassinet *et al.*, 2004). CyaA contributes to numerous pathological effects seen in the murine model of lung infection, such as efficient pulmonary colonization, induction of histopathological lesions in the lungs, recruitment of inflammatory leukocytes, and

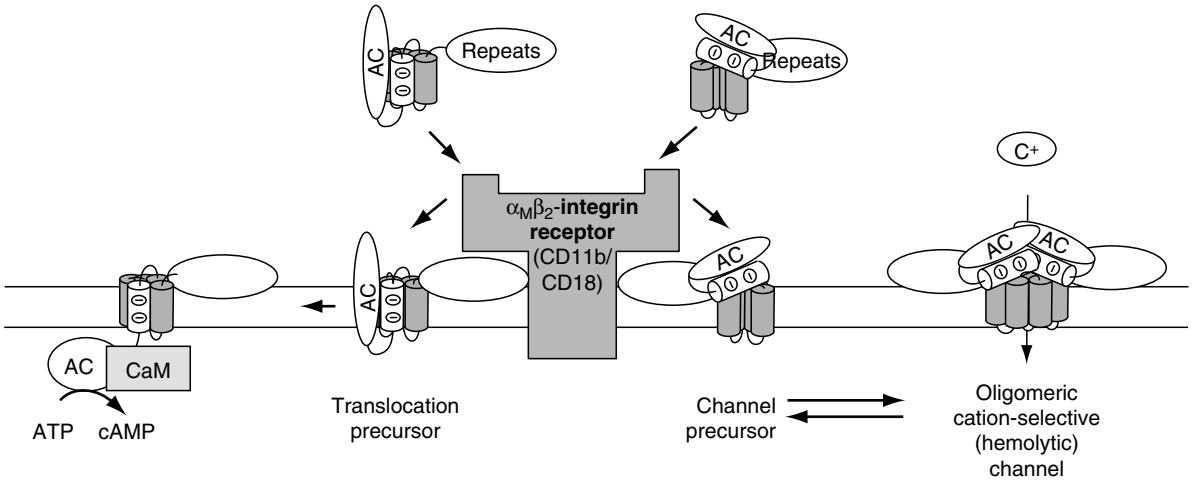



**FIGURE 16.2** Model of CyaA action on target cell membranes. The sum of currently available data suggests that following binding to the CD11b/CD18 receptor, CyaA inserts into the cell membrane in two different and more or less mutually exclusive conformations, one giving rise to a translocation precursor delivering the AC domain across the cytoplasmic membrane and the other inserting as a channel precursor and oligomerizing within the membrane to form the CyaA channel (Osickova *et al.*, 1999). The model is substantiated by a series of observations. Only about 50% of cell-associated AC activity is found translocated across a membrane into a trypsin-inaccessible compartment (Masin *et al.*, 2004). Different types of acylation differently affect only the hemolytic (channel-forming) activity of CyaA without affecting its capacity to deliver the AC domain across the membrane (Hackett *et al.*, 1995). Furthermore, amino acid substitutions can be generated in the channel domain that dissociate the AC delivery and channel-forming activities of CyaA (Osickova *et al.*, 1999) and can shift the balance between channel-forming and AC translocation activities of the toxin in both directions (Basler *et al.* unpublished).

induction of lethality (Weiss *et al.*, 1984; Gueirard *et al.*, 1998). In a physiologically relevant model of natural infection of mice by *B. bronchiseptica*, a mutant strain defective in CyaA production has been found to be completely avirulent in B and T cell deficient mice, while remaining fully virulent in neutropenic mice (Harvill *et al.*, 1999). This strongly suggests that myeloid phagocytic cells, and especially neutrophiles, are the primary *in vivo* targets of *B. bronchiseptica* CyaA and most likely also of the *B. pertussis* AC toxin (Weingart *et al.*, 2000). Indeed, CyaA-mediated intoxication has been found to inhibit signaling resulting from engagement of Fc receptors by opsonizing antibodies, thereby inhibiting phagocytosis of *B. pertussis* cells by neutrophils (Weingart and Weiss, 2000). CyaA activity further causes loss of chemotactic and oxidative burst capacities required for bactericidal activity of leukocytes (Friedman *et al.*, 1987). Moreover, the toxin can induce macrophage apoptosis (Khelef *et al.*, 1993; Khelef and Guiso, 1995) by a mechanism involving disruption of the membrane potential in mitochondria (Bachelet *et al.*, 2002).

## AC as a marker enzyme tool

The fact, that Gram-negative bacteria generally do not express CaM homologues and that in the absence of CaM the AC domain exhibits only residual enzyme activity allowed for the use of the AC domain as a reporter enzyme for tracing protein translocation into the eukaryotic cell cytosol (Sory and Cornelis, 1994). The AC reporter protein is fused to a bacterial test protein, such as a *Yersinia* Yop protein. While the hybrid protein exhibits very low AC activity in the bacterial cell and/or culture supernatants, once it is injected via the Yop effector domain into a eukaryotic host cell, the AC enzyme is activated by >1000-fold by the host CaM and catalyzes rapid conversion of ATP to cAMP. This can be easily assayed as intracellular cAMP accumulation that is proportional to the amount of injected AC fusion protein. This approach has become a standard technique to investigate mechanisms of protein translocation into eukaryotic host cells by the type III secretion apparatus.

A second application of AC activity makes use of the fact that the residual activity in the absence of CaM requires the physical interaction of the T25 and T18 fragments of the AC domain, which can be exploited to develop a bacterial two-hybrid system for detection of protein-protein interactions (Karimova *et al.*, 1998). When the T25 and T18 fragments are individually fused to peptides or proteins that are able to bind to each other, the interaction of the chimeric polypeptides yields a functional complementation of the T25 and

T18 fragments and the restoration of the residual capacity of the enzyme to convert intracellular ATP to cAMP. This, in turn, can be monitored in a rather sensitive manner by using *E. coli* lacking the endogenous adenylate cyclase activity as indicator strains for the two hybrid screening, as even very low levels of cAMP produced by the reconstituted AC enzyme still allow for the transcription of the genes involved in lactose and maltose catabolism in *E. coli*. Hence, this property offers a convenient detection system of functional interactions between proteins expressed in the two-hybrid screens on indicator plates or by the positive selection for growth of bacterial colonies in minimal media containing maltose as the unique carbon source (Dautin *et al.*, 2002).

### CyaA as a novel antigen delivery tool to induce specific T cell responses

The capacity of genetically detoxified CyaA to penetrate with high efficiency the professional antigen-presenting cells expressing CD11b/CD18 ($CD11b^{high}$) allowed for the development of CyaA into a tool for antigen delivery and induction of specific T cell responses against viruses and tumors (El-Azami-El-Idrissi *et al.*, 2002; Simsova *et al.*, 2004). Advantage can be taken of the capacity of the AC domain to accommodate foreign antigenic polypetides of up to 200 residues in length without losing cell invasiveness (Gmira *et al.*, 2001). Genetically detoxified hybrid CyaA toxoids can be used for highly efficient *in vivo* delivery of foreign $CD8^+$ and $CD4^+$ T cell epitopes or entire antigens into the MHC class I- and class II-dependent antigen-presentation pathways in parallel (Fayolle *et al.*, 2001; Schlecht *et al.*, 2004; Vordermeier *et al.*, 2004). In this manner strong $T_{h1}$-polarized and epitope-specific $CD4^+$ and $CD8^+$ CTL responses can be simultaneously induced for effective prophylactic vaccination against viruses (Saron *et al.*, 1997) or immunotherapy of tumors (Fayolle *et al.*, 1999; Dadaglio *et al.*, 2003). Phase I/II clinical trials in humans aiming at exploring this application of CyaA are currently in preparation.

## DERMONECROTIC TOXIN

In contrast to the other *Bordetella* protein toxins, dermonecrotic toxin (DNT) is an intracellular toxin (Cowell *et al.*, 1979). In fact, DNT was originally named endotoxin (Bordet and Gengou, 1909). It has also been called heat-labile toxin, because of its complete inactivation after 60 minutes. at 56°C (Livey and Wardlaw, 1984). The term DNT is due to the characteristic skin lesions that the toxin produces when injected into animals (Bordet and Gengou, 1909). Although its role in the pathogenesis is far from being clear, it is a very potent toxin, as an intravenous inoculation of very low doses of DNT is lethal for mice (Kume *et al.*, 1986).

### Structure-function relationship of DNT

DNT is produced by *B. pertussis, B. parapertussis*, and *B. bronchiseptica* (Parkhill *et al.*, 2003), and the degree of sequence similarities between the three proteins is very high (approximately 99% sequence identity). Its gene is also present in the *B. avium* genome, but the sequence of the *B. avium* DNT shows much divergence from the other three DNT sequences. The *B. pertussis* and the *B. bronchiseptica* DNT each contain 1,451 amino acids and have a predicted molecular mass of 159 kDa (Pullinger *et al.*, 1996; Walker and Weiss, 1994). The three-dimensional structure of DNT has not been determined, but based on the primary structure, the protein can be divided into two principal domains. The N-terminal domain constitutes the receptor-binding region, and the C-terminal domain expresses enzymatic activity. As such, DNT is thus a member of the A-B toxin family.

Within the N-terminal domain of DNT, a fragment consisting of the N-terminal 54 amino acid residues has been shown to inhibit the intoxication of cells by full-length DNT (Matsuzawa *et al.*, 2002). This 54 residues-long fragment is able to bind to the surface of target cells, and this binding can be inhibited by a monoclonal antibody that is able to neutralize the effects of DNT when mixed with the toxin. This antibody is not able to neutralize the toxin activity when microinjected into the target cells, indicating that it interferes with toxin binding. These observations indicate that the extreme N-terminal portion of DNT is crucial for receptor binding. Interestingly, the 54 residue-long peptide does not bind to DNT-resistant cells, indicating that DNT-sensitive cells contain on their surface a specific receptor for the toxin. This receptor remains to be identified.

The C-terminal, catalytic domain of DNT shares sequence similarities with the *E. coli* cytotoxic necrotizing factor (CNF) (Walker and Weiss, 1994). Both toxins cause deamidation of glutamine 63 in Rho (Horiguchi *et al.*, 1997; Flatau *et al.*, 1997). In the presence of primary amines, DNT also catalyzes the transglutamination of Rho. This catalytic activity is expressed by a fragment covering residues 1136–1451 of DNT (Schmidt *et al.*, 1999), the region that is highly homologous to the catalytic domain of CNF1. The three dimensional structure of the CNF1 catalytic domain has been determined at a 1.83 Å resolution (Buetow *et al.*, 2001) and was found to contain a Cys-His-main chain oxygen catalytic triad. This triad is highly conserved

among the homologous proteins, suggesting that they all express their enzymatic activity by the same mechanism. The catalytic cysteine of CNF1 is located at the base of a deep pocket. This position would be expected to restrict the access to potential substrates, thereby explaining the high specificity of these toxins. Whereas the catalytic pocket of CNF1 is electrostatically nondescript, the DNT pocket carries a net negative charge that may attract positively charged primary amines. This structural difference may account for the differences in enzymatic activities between the two toxins, since CNF1 is preferentially a deamidase, whereas DNT is preferentially a transglutaminase, replacing the side chain of amide of the glutamine by a primary amine rather than by a hydroxyl (Smidt *et al*., 1999). Site-directed alterations of the conserved Cys-1292 in DNT abolish both the deamidase and transglutaminase activities of DNT.

## Molecular mechanisms of DNT action

DNT is produced by *Bordetella* as a single polypeptide chain. However, upon binding to its target cell receptor, it can undergo proteolytic cleavage, which separates the N-terminal fragment from the catalytic domain. The mammalian endoprotease furin has been shown to cleave DNT specifically at the C-terminal site of Arg-44 (Matsuzawa *et al*., 2004). This cleavage appears to be necessary for the expression of toxin activity, as mutant toxins that are insensitive to furin have no toxic effect. Conversely, an *in vitro* nicked form of the toxin is more potent than the intact form, indicating that nicking by furin is a prerequisite for toxin action. The nicked toxin part lacking the first 44 amino acid residues is then able to dissociate from the receptor-binding domain and to cross the lipid bilayer to gain access to the cytosol, where the catalytic domain is able to express its enzyme activity. The translocation step of DNT relies on amino acid residues 45–166, which include a putative transmembrane domain. Translocation does not require acidification of endosomes, nor retrograde vesicular transport. However, the toxin appears to be internalized by dynamin-dependent endocytosis.

Once the toxin has reached the cytoplasm of the target cells, its enzymatic domain catalyzes the deamidation of the small GTPases Rho (Horiguchi *et al*., 1997), Rac, and Cdc42 (Masuda *et al*., 2002). Upon deamidation Rho, Rac, and Cdc42 lose their GTP-hydrolyzing, but not their GTP-binding, activities. Reduced GTP hydrolysis results in prolonged activation of these small GTP-binding proteins, which in their activated states interact with their respective effectors. This, in turn, results in ultrastructural rearrangements of the cytoskeleton, leading to morphological changes, such as the formation of lamellipodia and filopodia, accompanied by the assembly of actin stress fibers and focal adhesions (Horiguchi *et al*., 1995).

In addition to Rho, Rac, and Cdc42 deamidation, DNT also catalyzes cross-linking of Rho, Rac, and Cdc42 with ubiquitous polyamines (Masuda *et al*., 2000). Polyamination occurs at the same residues as deamidation and also results in reduced GTP-hydrolase activity, without affecting GTP binding. Interestingly, polyaminated Rho, even in its GDP-bound form, interacts more effectively with its effector ROCK than deamidated Rho in the GTP-bound form, indicating that polyamination of Rho transduces signals to its effector ROCK in a GTP-independent manner.

The DNT-catalyzed modification of Rho also induces the proliferation of cytoplasmic membrane organelles, such as the Golgi apparatus, the endoplasmic reticulum, and the mitochondria, as well as the formation of plasmalemmal calveolae (Senda *et al*., 1997). The focal adhesion phenotype may be related to the stimulation of tyrosine phosphorylation of focal adhesion kinase and paxillin upon modification of Rho (Lacerda *et al*., 1997).

## Role of DNT in pathogenesis

One of the principal target cells for DNT is the osteoblast. Upon treatment with DNT, osteoblasts undergo a transition from a spindle-shaped, stretched morphology to spherical or block forms (Horiguchi *et al*., 1991). In addition, they lose their extensions and form small blebs on their surface, although they remain viable and continue to proliferate. Alkaline phosphatase activity and the accumulation of type I collagen are reduced in treated cells compared to control cells, suggesting that DNT impairs the ability of osteoblasts to differentiate, and that thereby the toxin acts on osteogenesis.

The pathology induced by DNT has best been studied in pigs infected with *B. bronchiseptica*, where the toxin has been shown to induce ultrastructural changes in the nasal turbinate bones (Silvera *et al*., 1982), leading to the development of atrophic rhinitis. The use of *B. bronchiseptica* strains differing in their ability to produce DNT in a neonatal swine infection model has indicated that only strains that synthesize high levels of DNT are able to produce nasal and lung lesions (Roop *et al*., 1987). These lesions vary from moderate to severe, establishing a direct correlation between the level of toxin production and the ability of *B. bronchiseptica* to induce nasal and lung lesions. In fact, mutant derivatives of virulent strains that lack DNT do not induce clinical manifestations, but have been

shown to induce protective immunity. DNT-deficient strains may, therefore, be considered as live vaccine candidates against atrophic rhinitis (Nagano *et al*., 1988).

The role of the *B. bronchiseptica* DNT in the pathogenesis of respiratory disease in swine has more recently been firmly established by the use of isogenic strains only differing in the production of DNT (Brockmeier *et al*., 2002). Compared to the wild-type strain, the DNT-deficient mutant was found to slightly less well colonize the upper respiratory tract of one-week-old piglets. The colonization ability of the lower respiratory tract was not affected by the lack of DNT. In addition to the slight difference in upper respiratory tract colonization, the mutant strain did not induce turbinate atrophy or pneumonia in the piglets, whereas turbinate atrophy and bronchopneumonia were found in most piglets infected with the parent strain. Similar observations have been made in the mouse model after infection with either the wild-type or the isogenic mutant strain (Magyar *et al*., 2000).

The role, if any, of DNT in whooping cough is less well understood. *B. pertussis* strains lacking DNT are as virulent in infant mice as the wild-type parent strain (Weiss and Goodwin, 1989), and different human isolates from whooping cough patients may vary in their ability to produce the toxin. There is apparently no correlation between the level of DNT production and the infectivity of *B. pertussis* in humans. In fact, most clinical isolates appear not to produce DNT, suggesting that this toxin does not play a major role in the virulence of *B. pertussis* in humans (Gueirard and Guiso, 1993).

## REFERENCES


Agarwal, R.K., Sun, S.H., Su, S.B., Chan, C.C. and Caspi, R.R. (2002). Pertussis toxin alters the innate and the adaptive immune responses in a pertussis-dependent model of autoimmunity. *J. Neuroimmunol*. **129,** 133–140.

Antoine, R. and Locht, C. (1990). The role of the disulfide bond and the carboxy-terminal region of the S1 subunit in the assembly and biosynthesis of pertussis toxin. *Infect. Immun*. **58,** 1518–1526.

Antoine, R. and Locht, C. (1994). The NAD-glycohydrolase activity of the pertussis toxin S1 subunit: involvement of the catalytic His-35 residue. *J. Biol. Chem*. **269,** 6450–6457.

Antoine, R. Tallett, A., van Heyningen, S. and Locht, C. (1993). Evidence for a catalytic role of Glu 129 in the NAD-glycohydrolase activity of the pertussis toxin S1 subunit. *J. Biol. Chem*. **268,** 24149–24155.

Antoine, R., Raze, D. and Locht, C. (1996). Genetic analysis of the pertussis toxin locus. *Zbl. Bakt. Suppl*. **28,** 44–45.

Armstrong, G.D., Howard, L.A. and Peppler, M.S. (1988). Use of glycosyltransferases to restore pertussis toxin receptor activity to asialogalactofetuin. *J. Biol. Chem*. **263,** 8677–8684.

Bachelet, M., Richard, M.J., Francois, D. and Polla, B.S. (2002). Mitochondrial alterations precede *Bordetella pertussis*-induced apoptosis. *FEMS Immunol. Med. Microbiol*. **32,** 125–131.

Bagley, K.C., Abdelwahab, S.F., Tuskan, R.G., Fouts, T.R. and Lewis, G.K. (2002). Pertussis toxin and the adenylate cyclase toxin from *Bordetella pertussis* activate human monocyte-derived dendritic cells and dominantly inhibit cytokine production through a cAMP-dependent pathway. *J. Leukoc. Biol*. **72,** 962–969.

Baker, S.M., Masi, A., Liu, D.-F., Novitsky, B.K. and Deich, R.A. (1995). Pertussis toxin export genes are regulated by the *ptx* promoter and may be required for efficient translation of *ptx* mRNA in *Bordetella pertussis*. *Infect. Immun*. **63,** 3920–3926.

Baron, C., O'Callaghan, D. and Lanka, E. (2002). Bacterial secrets of secretion: EuroConference on the biology of type IV secretion processes. *Mol. Microbiol*. **43,** 1359–1365.

Barry, E.M., Weiss, A.A., Ehrmann, I.E., Gray, M.C., Hewlett, E.L. and Goodwin, M.S. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cyaC*, for activation. *J. Bacteriol*. **173,** 720–726.

Basar, T., Havlicek, V., Bezouskova, S., Hackett, M. and Sebo, P. (2001). Acylation of lysine 983 is sufficient for toxin activity of *Bordetella pertussis* adenylate cyclase. Substitutions of alanine 140 modulate acylation site selectivity of the toxin acyltransferase CyaC. *J. Biol. Chem*. **276,** 348–354.

Bassinet, L., Fitting, C., Housset, B., Cavaillon, J.M. and Guiso, N. (2004). *Bordetella pertussis* adenylate cyclase-hemolysin induces interleukin-6 secretion by human tracheal epithelial cells. *Infect. Immun*. **72,** 5530–5533.

Baumann, U., Wu, S., Flaherty, K.M. and McKay, D.B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. *EMBO J.* **12,** 3357–3364.

Bejerano, M., Nisan, I., Ludwig, A., Goebel, W. and Hanski, E. (1999). Characterization of the C-terminal domain essential for toxic activity of adenylate cyclase toxin. *Mol. Microbiol*. **31,** 381–392.

Belcher, C.E., Drenkow, J., Kehoe, B., Gingeras, T.R., McNamara, N., Lemjabbar, H., Basbaum, C. and Relman, D.A. (2000). The transcriptional responses of respiratory epithelial cells to *Bordetella pertussis* reveal host defensive and pathogen counter-defensive strategies. *Proc. Natl. Acad. Sci. USA* **97,** 13847–13852.

Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C. and Ullmann, A. (1990). Deletions affecting hemolytic and toxin activities of *Bordetella pertussis* adenylate cyclase. *Infect. Immun*. **58,** 3242–3247.

Benz, R., Maier, E., Ladant, D., Ullmann, A. and Sebo, P. (1994). Adenylate cyclase toxin (CyaA) of *Bordetella pertussis*. Evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli*. *J. Biol. Chem*. **269,** 27231–27239.

Betsou, F., Sebo, P. and Guiso, N. (1993). CyaC-mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase-hemolysin. *Infect. Immun*. **61,** 3583–3589.

Bogdan, J.A., Yuan, W., Long-Rowe, K.O., Sarwar, J., Brucker, E.A. and Blake, M.S. (2003). Identification of peptides that mimic the pertussis toxin binding site on bovine fetuin. *Appl. Environ. Microbiol*. **69,** 6272–6279.

Bordet, J. and Gengou, O. (1909). L'endotoxine coquelucheuse. *Ann. Inst. Pasteur (Paris)* **23,** 415–419.

Bouhss, A., Krin, E., Munier, H., Gilles, A.M., Danchin, A., Glaser, P. and Barzu, O. (1993). Cooperative phenomena in binding and activation of *Bordetella pertussis* adenylate cyclase by calmodulin. *J. Biol. Chem*. **268,** 1690–1694.

Brennan, M.J., David, J.L., Kenomer, J.G. and Manclark, C.R. (1988). Binding of pertussis toxin to a 165-kilodalton Chinese hamster ovary cell glycoprotein. *J. Biol. Chem*. **263,** 4895–4899.

Brockmeier, S.L., Register, K.B., Magyar, T., Lax, A.J., Pullinger, G.D. and Kunkle, R. A. (2002). Role of the dermonecrotic toxin of *Bordetella bronchiseptica* in the pathogenesis of respiratory disease in swine. *Infect. Immun*. **70,** 481–490.

Buetow, L., Flatau, G., Chiu, K., Boquet, P. and Ghosh, P. (2001). Structure of the Rho-activating domain of *Escherichia coli* cytotoxic necrotizing factor 1. *Nat. Struct. Biol*. **8,** 584–588.

Burns, D.L., Fiddner, S., Cheung, A.M. and Verma, A. (2004). Analysis of subassemblies of pertussis toxin subunits *in vivo* and their interaction with the ptl transport apparatus. *Infect. Immun*. **72,** 5365–5372.

Castro, M.G., McNamara, U. and Carbonetti, N.H. (2001). Expression, activity, and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. *Cell Microbiol*. **3,** 45–54.

Cheung, A.M., Farizo, K.M. and Burns, D.L. (2004). Analysis of relative levels of production of pertussis toxin subunits and Ptl proteins in Bordetella pertussis. *Infect. Immun*. **72,** 2057–2066.

Clark, C.G. and Armstrong, G.F. (1990). Lymphocyte receptors for pertussis toxin. *Infect. Immun*. **58,** 3840–3846.

Confer, D.L. and Eaton, J.W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* **217,** 948–950.

Cook, D.M., Farizo, K.M. and Burns, D.L. (1999). Identification and characterization of PtlC, an essential component of the pertussis toxin secretion system. *Infect. Immun*. **67,** 754–759.

Cortina, G, Krueger, K.M. and Barbieri, J.T. (1991). The carboxyl terminus of the S1 subunit of pertussis toxin confers high affinity binding to transducin. *J. Biol. Chem*. **266,** 23810–23814.

Cortina, G. and Barbieri, J.T. (1989). Role of tryptophan 26 in the NAD glycohydrolase reaction of the S-1 subunit of pertussis toxin. *J. Biol. Chem*. **264,** 17322–17328.

Cowell, J.L., Hewlett, E.L. and Manclark, C.R. (1979). Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*. *Infect. Immun*. **25,** 896–901.

Craig-Mylius, K.A. and Weiss, A.A. (1999). Mutants in the ptlA-H genes of Bordetella pertussis are deficient for pertussis toxin secretion. *FEMS Microbiol. Lett*. **179,** 479–484.

Craig-Mylius, K.A,, Stenson, T.H. and Weiss, A.A. (2000). Mutations in the S1 subunit of pertussis toxin that affect secretion. *Infect Immun*. **68,** 1276–1281.

Dadaglio, G., Morel, S., Bauche, C., Moukrim, Z., Lemonnier, F.A., Van Den Eynde, B.J., Ladant, D. and Leclerc, C. (2003). Recombinant adenylate cyclase toxin of *Bordetella pertussis* induces cytotoxic T lymphocyte responses against HLA*0201-restricted melanoma epitopes. *Int. Immunol*. **15,** 1423–1430.

Dautin, N., Karimova, G. and Ladant, D. (2002). *Bordetella pertussis* adenylate cyclase toxin: a versatile screening tool. *Toxicon* **40,** 1383–1387.

Decker, M.D. and Edwards, K.M. (2000). Acellular pertussis vaccines. *Pediatr. Clin. North Am*. **47,** 309–335.

Drum, C.L., Yan, S.Z., Bard, J., Shen, Y.Q., Lu, D., Soelaiman, S., Grabarek, Z., Bohm, A. and Tang, W.J. (2002). Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. *Nature* **415,** 396–402.

Ehrmann, I.E., Gray, M.C., Gordon, V.M., Gray, L.S. and Hewlett, E.L. (1991). Hemolytic activity of adenylate cyclase toxin from *Bordetella pertussis*. *FEBS Lett*. **278,** 79–83.

El-Azami-El-Idrissi, M., Bauche, C., Loucka, J., Osicka, R., Sebo, P., Ladant, D. and Leclerc, C. (2003). Interaction of *Bordetella pertussis* adenylate cyclase with CD11b/CD18: Role of toxin acylation and identification of the main integrin interaction domain. *J. Biol. Chem*. **278,** 38514–38521.

El-Azami-El-Idrissi, M., Ladant, D. and Leclerc, C. (2002). The adenylate cyclase of *Bordetella pertussis*: a vector to target antigen presenting cells. *Toxicon* **40,** 1661–1665.

El Baya, A., Linnemann, R., von Olleschik-Elbheim, L. and Schmidt, M.A. (1995). Identification of binding proteins for pertussis toxin on pancreatic a β cell-derived insulin-secreting cells. *Microb. Pathog*. **18,** 173–185.

El Baya, A., Linnemann, R., von Olleschik-Elbheim, L., Robenek, H. and Schmidt, M.A. (1997). Endocytosis and retrograde transport of pertussis toxin to the Golgi complex as a prerequisite for cellular intoxication. *Eur. J. Cell Biol*. **73,** 40–48.

Farizo, K.M., Cafarella, T.G. and Burns, D.L. (1996). Evidence for a ninth gene, ptlI, in the locus encoding the pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *J. Biol. Chem*. **271,** 31643–31649.

Farizo, K.M., Huang, T. and Burns, D.L. (2000). Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from *Bordetella pertussis*. *Infect. Immun*. **68,** 4049–4054.

Farizo, K.M., Fiddner, S., Cheung, A.M. and Burns, D.L. (2002). Membrane localization of the S1 subunit of pertussis toxin in *Bordetella pertussis* and implications for pertussis toxin secretion. *Infect. Immun*. **70,** 1193–1201.

Fayolle, C., Ladant, D., Karimova, G., Ullmann, A. and Leclerc, C. (1999). Therapy of murine tumors with recombinant *Bordetella pertussis* adenylate cyclase toxins carrying a cytotoxic T-cell epitope. *J. Immunol*. **162,** 4157–4162.

Fayolle, C., Osickova, A., Osicka, R., Henry, T., Rojas, M.J., Saron, M.F., Sebo, P. and Leclerc, C. (2001). Delivery of multiple epitopes by recombinant detoxified adenylate cyclase of *Bordetella pertussis* induces protective antiviral immunity. *J. Virol*. **75,** 7330–7338.

Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387,** 729–733.

Friedman, R.L., Fiederlein, R.L., Glasser, L. and Galgiani, J.N. (1987). *Bordetella pertussis* adenylate cyclase: Effects of affinity purified adenylate cyclase on human polymorphonucleare leukocyte function. *Infect. Immun*. **55,** 135–140.

Gentile, F., Raptis, A., Knipling, L.G. and Wolff, J. (1988). *Bordetella pertussis* adenylate cyclase. Penetration into host cells. *Eur. J. Biochem*. **175,** 447–453.

Glaser, P., Elmaoglou-Lazaridou, A., Krin, E., Ladant, D., Bârzu, O. and Danchin, A. (1989). Identification of residues essential for catalysis and binding of calmodulin in *Bordetella pertussis* adenylate cyclase by site-directed mutagenesis. *EMBO J*. **8,** 967–972.

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A. and Danchin, A. (1988a). The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. *Mol. Microbiol*. **2,** 19–30.

Glaser, P., Munier, H., Gilles, A.M., Krin, E., Porumb, T., Bârzu, O., Sarfati, R., Pellecuer, C. and Danchin, A. (1991). Functional consequences of single amino acid substitutions in calmodulin-activated adenylate cyclase of *Bordetella pertussis*. *EMBO J.* **10,** 1683–1688.

Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A. and Danchin, A. (1988b). Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-hemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J.* **7,** 3997–4004.

Gmira, S., Karimova, G. and Ladant, D. (2001). Characterization of recombinant *Bordetella pertussis* adenylate cyclase toxins carrying passenger proteins. *Res. Microbiol*. **152,** 889–900.

Goodwin, M.S. and Weiss, A.A. (1990). Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infect. Immun*. **58,** 3445–3447.

Gordon, V.M., Leppla, S.H. and Hewlett, E.L. (1988). Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus*

*anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infect. Immun*. **56,** 1066–1069.

Gray, M., Szabo, G., Otero, A., Gray, L. and Hewlett, E. (1998). Distinct mechanisms for $K^+$ efflux, intoxication, and hemolysis by *Bordetella pertussis* AC toxin. *J. Biol. Chem*. **273,** 18260–18267.

Gray, M.C., Donato, G.M., Jones, F.R., Kim, T. and Hewlett, E.L. (2004). Newly secreted adenylate cyclase toxin is responsible for intoxication of target cells by *Bordetella pertussis*. *Mol. Microbiol*. **53,** 1709–1719.

Gueirard, P. and Guiso, N. (1993). Virulence of *Bordetella bronchiseptica*: role of adenlyate cyclase. *Infect. Immun*. **61,** 4072–4078.

Gueirard, P., Druilhe, A., Pretolani, M. and Guiso, N. (1998). Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo*. *Infect. Immun*. **66,** 1718–1725.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the alpha(M)beta(2) Integrin (CD11b/CD18). *J. Exp. Med*. **193,** 1035–1044.

Guermonprez, P., Ladant, D., Karimova, G., Ullmann, A. and Leclerc, C. (1999). Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. *J. Immunol*. **162,** 1910–1916.

Hackett, M., Guo, L., Shabanowitz, J., Hunt, D.F. and Hewlett, E.L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis*. *Science* **266,** 433–435.

Hackett, M., Walker, C.B., Guo, L., Gray, M.C., Van, C.S., Ullmann, A., Shabanowitz, J., Hunt, D.F., Hewlett, E.L. and Sebo, P. (1995). Hemolytic, but not cell-invasive activity, of adenylate cyclase toxin is selectively affected by differential fatty-acylation in *Escherichia coli*. *J. Biol. Chem*. **270,** 20250–20253.

Hanski, E. (1989). Invasive adenylate cyclase toxin of *Bordetella pertussis*. *Trends Biochem. Sci*. **14,** 459–463.

Hanski, E. and Farfel, Z. (1985). *Bordetella pertussis* invasive adenylate cyclase: partial resolution and properties of its cellular penetration. *J. Biol. Chem*. **260,** 5526–5532.

Harvill, E.T., Cotter, P.A., Yuk, M.H. and Miller, J.F. (1999). Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating host immunity. *Infect. Immun*. **67,** 1493–1500.

Hausman, S.Z. and Burns, D.L. (1992). Interaction of pertussis toxin with cells and model membranes. *J. Biol. Chem*. **267,** 13735–13739.

Hausman, S.Z. and Burns, D.L. (1993). Binding of pertussis toxin to lipid vesicles containing glycolipids. *Infect. Immun*. **61,** 335–337.

Havlicek, V., Higgins, L., Chen, W., Halada, P., Sebo, P., Sakamoto, H. and Hackett, M. (2001). Mass spectrometric analysis of recombinant adenylate cyclase toxin from *Bordetella pertussis* strain 18323/pHSP9. *J. Mass Spectrom*. **36,** 384–391.

Hazes, B., Boodhoo, A., Cockle, S.A. and Read R.J. (1996). Crystal structure of the pertussis toxin-ATP complex: a molecular sensor. *J. Mol. Biol*. **258,** 661–671.

Hazes, B. and Read, R.J. (1997). Accumulating evidence suggests that several A-B toxins subvert the ER-associated protein degradation pathway to enter target cells. *Biochemistry* **37,** 11051–11054.

He, J., Gurunathan, S., Iwasaki, A., Ash-Shaheed, B. and Kelsall, B.L. (2000). Primary role for Gi protein signaling in the regulation of interleukin 12 production and the induction of T helper cell type 1 responses. *J. Exp. Med*. **191,** 1605–1610.

Hewlett, E.L., Gray, L., Allietta, M., Ehrmann, I.E., Gordon, V.M. and Gray, M.C. (1991). Adenylate cyclase toxin from *Bordetella pertussis*. Conformational change associated with toxin activity. *J. Biol. Chem*. **266,** 17503–17508.

Hewlett, E.L., Urban, M.A., Manclark, C.R. and Wolff, J. (1976). Extracytoplasmic adenylate cyclase of *Bordetella pertussis*. *Proc. Natl. Acad. Sci. USA* **73,** 1926–1930.

Horiguchi, Y., Inoue, N., Masuda, M., Kashimoto, T., Katahira, J., Sugimoto, N. and Matsuda, M. (1997). *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln-63 of the GTP-binding protein Rho. *Proc. Natl. Acad. Sci. USA* **94,** 11623–11626.

Horiguchi, Nakai, T. and Kume, K. (1991). Effects of *Bordetella bronchiseptica* dermonecrotic toxin on the structure and function of osteoblastic clone MC3T3-E1 cells. *Infect. Immun*. **59,** 1112–1116.

Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. and Matsuda, M. (1995). *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein Rho. *J. Cell Sci*. **108,** 3243–3251.

Hou, W., Wu, Y., Sun, S., Shi, M., Sun, Y., Yang, C., Pei, G., Gu, Y., Zhong, C. and Sun, B. (2003). Pertussis toxin enhances Th1 responses by stimulation of dendritic cells. *J. Immunol*. **170,** 1728–1736.

Ishibashi, Y. and Nishikawa, A. (2002). Bordetella pertussis infection of human respiratory epithelial cells up-regulates intercellular adhesion molecule-1 expression: role of filamentous hemagglutinin and pertussis toxin. *Microb. Pathog*. **33,** 115–125.

Iwaki, M., Ullmann, A. and Sebo, P. (1995). Identification by *in vitro* complementation of regions required for cell-invasive activity of *Bordetella pertussis* adenylate cyclase toxin. *Mol. Microbiol*. **17,** 1015–1024.

Johnson, F.D. and Burns, D.L. (1994). Detection and subcellular localization of three Ptl proteins involved in the secretion of pertussis toxin from *Bordetella pertussis*. *J. Bacteriol*. **176,** 5350–5356.

Karimova, G., Pidoux, J., Ullmann, A. and Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. USA* **95,** 5752–5756.

Katada, T. and Ui, M. (1982). Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. USA* **79,** 3129–3133.

Khelef, N. and Guiso, N. (1995). Induction of macrophage apoptosis by *Bordetella pertussis* adenylate cyclase-hemolysin. *FEMS Microbiol. Lett*. **134,** 27–32.

Khelef, N., Sakamoto, H. and Guiso, N. (1992). Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. *Microb. Pathog*. **12,** 227–235.

Khelef, N., Zychlinsky, A. and Guiso, N. (1993). *Bordetella pertussis* induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. *Infect. Immun*. **61,** 4064–4071.

Knapp, O., Maier, E., Polleichtner, G., Masin, J., Sebo, P. and Benz, R. (2003). Channel formation in model membranes by the adenylate cyclase toxin of *Bordetella pertussis*: effect of calcium. *Biochemistry* **42,** 8077–8084.

Koronakis, V., Eswaran, J. and Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Ann. Rev. Biochem*. **73,** 467–489.

Kotob, S.I. and Burns, D.L. 1997. Essential role of the consensus nucleotide-binding site of PtlH in secretion of pertussis toxin from *Bordetella pertussis*. *J. Bacteriol*. **179,** 7577–7580.

Kotob, S.I., Hausman, S.Z. and Burns, D.L. (1995). Localization of the promoter for the *ptl* genes of Bordetella pertussis, which encode proteins essential for secretion of pertussis toxin. *Infect. Immun*. **63,** 3227–3230.

Kume, K., Nakai, T., Samejima, Y. and Sugimoto, C. (1986). Properties of dermonecrotic toxin prepared from sonic extracts of *Bordetella bronchiseptica*. *Infect. Immun*. **52,** 370–377.

Lacerda, H.M., Pullinger, G.D., Lax, A.J. and Rozengurt, E. (1997). Cytotoxic necrotizing factor 1 from Escherichia coli and dermonecrotic toxin from *Bordetella bronchiseptica* induce p21(rho)-dependent tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 cells. *J. Biol. Chem*. **272,** 9587–9596.

Ladant, D. (1988). Interaction of *Bordetella pertussis* adenylate cyclase with calmodulin: identification of two separated calmodulin-binding domains. *J. Biol. Chem*. **263,** 2612–2618.

Ladant, D., Glaser, P. and Ullmann, A. (1992). Insertional mutagenesis of *Bordetella pertussis* adenylate cyclase. *J. Biol. Chem*. **267,** 2244–2250.

Ladant, D., Michelson, S., Sarfati, R.S., Gilles, A.-M., Predeleanu, R. and Bârzu, O. (1989). Characterization of the calmodulin-binding and of the catalytic domains of *Bordetella pertussis* adenylate cyclase. *J. Biol. Chem*. **264,** 4015–4020.

Laoide, B.M. and Ullmann, A. (1990). Virulence dependent and independent regulation of the *Bordetella pertussis cya* operon. *EMBO J*. **9,** 999–1005.

Livey, I. and Wardlaw, A. C. (1984). Production and properties of *Bordetella pertussis* heat labile toxin. *J. Med. Microbiol*. **17,** 91–103.

Lobet, Y., Feron, C., Dequesne, G., Simoen, E., Hauser, P. and Locht, C. (1993). Site-specific alterations in the B-oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J. Exp. Med*. **177,** 79–87.

Locht, C. (1999). Molecular aspects of *Bordetella pertussis* pathogenesis. *Internatl. Microbiol*. **2,** 137–144.

Locht, C. and Antoine, R. (1995). A proposed mechanism of ADP-ribosylation catalyzed by the pertussis toxin S1 subunit. *Biochimie* **77,** 333–340.

Locht, C. and Keith, J.M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232,** 1258–1264.

Locht, C., Antoine, R., Veithen, A. and Raze, D. (2000). Pertussis toxin: Structure-function relationship. In: *Handbook of Experimental Pharmacology,* Vol 145 : Bacterial Protein Toxins. (eds. K. Aktories I. Just), Springer Verlag, Berlin, pp. 167–185.

Locht, C., Antoine, R. and Jacob-Dubuisson, F. (2001). *Bordetella pertussis*, Molecular pathogenesis under multiple aspects. *Cur. Opin. Microbiol*. **4,** 82–89.

Locht, C., Lobet, Y., Feron, C., Cieplak, W. and Keith, J. M. (1990). The role of cysteine 41 in the enzymatic activities of the perutssis toxin S1 subunit as investigated by site-directed mutagenesis. *J. Biol. Chem*. **265,** 4552–4559.

Loosmore, S., Zealey, G., Cockle, S., Boux, H., Chong, P., Yacoob, R. and Klein, M. (1993). Characterization of pertussis toxin analogs containing mutations in B-oligomer subunits. *Infect. Immun*. **61,** 2316–2324.

Magyar, T., Glavits, R., Pullinger, G.D. and Lax, A.J. (2000). The pathological effect of the *Bordetella* dermonecrotic toxin in mice. *Acta Vet. Hung*. **48,** 397–406.

Masin, J., Konopasek, I., Svobodova, J. and Sebo, P. (2004). Different structural requirements for adenylate cyclase toxin interactions with erythrocyte and liposome membranes. *Biochim. Biophys. Acta* **1660,** 144–154.

Masuda, M., Betancourt, L., Matsuzawa, T., Kashimoto, T., Takao, T., Shimonishi, Y. and Horiguchi, Y. (2000). Activation of rho through a cross-link with polyamines catalyzed by *Bordetella* dermonecrotizing toxin. *EMBO J.* **19,** 521–530.

Masuda, M., Minami, M., Shime, H., Matsuzawa, T. and Horiguchi Y. (2002). *In vivo* modifications of small GTPase Rac and Cdc42 by *Bordetella* dermonecrotic toxin. *Infect. Immun*. **70,** 998–1001.

Matsuzawa, T., Fukui, A., Kashimoto, T., Nagao, K., Oka, K., Miyake, M. and Horiguchi, Y. (2004). *Bordetella* dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. *J. Biol. Chem*. **279,** 2866–2872.

Matsuzawa, T., Kashimoto, T., Katahira, J. and Horiguchi, Y. (2002). Identification of a receptor-binding domain of *Bordetella* dermonecrotic toxin. *Infect. Immun*. **70,** 3427–3432.

Mielcarek, N., Riveau, G., Remoué, F., Antoine, R., Capron, A. and Locht, C. (1998). Homologous and heterologous protection after single intranasal administration of live attenuated recombinant *Bordetella pertussis*. *Nat. Biotechnol*. **16,** 454–457.

Munier, H., Bouhss, A., Krin, E., Danchin, A., Gilles, A.M., Glaser, P. and Barzu, O. (1992). The role of histidine 63 in the catalytic mechanism of *Bordetella pertussis* adenylate cyclase. *J. Biol. Chem*. **267,** 9816–9820.

Munoz, J.J. (1985). Biological activities of pertussigen (pertussis toxin). In: *Pertussis Toxin* (eds. R.D. Sekura, J. Moss, and M. Vaughn), pp. 1–18. Academic Press, Orlando, FL.

Nagano, H., Nakai, T., Horiguchi, Y. and Kume, K. (1988). Isolation and characterization of mutant strains of *Bordetella bronchispetica* lacking dermonecrotic toxin-producing ability. *J. Clin. Microbiol*. **26,** 1983–1987.

Nicosia, A., Perugini, M., Franzini, C., Casagli, M.C., Borri, M.G., Antoni, G., Almoni, M., Neri, P., Ratti, G. and Rappuoli, R. (1986). Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proc. Natl. Acad. Sci. USA* **83,** 4631–4635.

Osickova, A., Osicka, R., Meyer, E., Benz, R. and Sebo, P. (1999). An amphipathic α-helix including glutamates 509 and 516 is crucial for membrane translocation of adenylate cyclase toxin and modulates formation and cation selectivity of its membrane channels. *J. Biol. Chem*. **274,** 37644–37650.

Otero, A.S., Yi, X., Gray, M.C., Szabo, G. and Hewlett, E.L. (1995). Membrane depolarization prevents cell invasion by *Bordetella pertussis* adenylate cyclase toxin. *J. Biol. Chem*. **270,** 9695–9697.

Otto, T., Lummen, G., Kalble, T., Recker, F., Krege, S., Bex, A., Noll, F. and Rubben, H. (1999). Intravesical therapy with pertussis toxin before radical cystectomy in patients with bladder cancer: a Phase I study. *Urology* **54,** 458–460.

Pallen, M.J., Lam, A.C., Loman, N.J. and McBride, A. (2001). An abundance of bacterial ADP-ribosyltransferases—implications for the origin of exotoxins and their human homologues. *Trends Microbiol*. **9,** 302–307.

Parkhill, J., Sebaihia, M., Preston, A., Murphy, L.D., Thomson, N., Harris, D.E., Holden, M.T., Churcher, C.M., Bentley, S.D., Mungall, K.L., Cerdeno-Tarraga, A.M., Temple, L., James, K., Harris, B., Quail, M.A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A., Davis, P., Doggett, J., Feltwell, T., Goble, A., Hamlin, N., Hauser, H., Holroyd, S., Jagels, K., Leather. S., Moule, S., Norberczak, H., O'Neil, S., Ormond, D., Price, C., Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B.G. and Maskell, D.J. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis, Bordetella parapertussis*, and *Bordetella bronchiseptica*. *Nat. Genet*. **35,** 32–40.

Pittman, M. (1984). The concept of pertussis as a toxin-mediated disease. *Pediatr. Infect. Dis*. **3,** 467–486.

Pullinger, G.D., Adams, T.E., Mullan, P.B., Garrod, T.I. and Lax, A.J. (1996). Cloning, expression, and molecular characterization of the dermonecrotic toxin of *Bordetella* spp. *Infect. Immun*. **64,** 4163–4171.

Rambow-Larsen, A.A. and Weiss, A.A. (2004). Temporal expression of pertussis toxin and Ptl secretion proteins by *Bordetella pertussis*. *J. Bacteriol*. **186,** 43–50.

Rambow-Larsen, A.A. and Weiss, A.A. (2002). The PtlE protein of *Bordetella pertussis* has peptidoglycanase activity required for Ptl-mediated pertussis toxin secretion. *J. Bacteriol*. **184,** 2863–2869.

Rhodes, C.R., Gray, M.C., Watson, J.M., Muratore, T.L., Kim, S.B., Hewlett, E.L. and Grisham, C.M. (2001). Structural consequences of divalent metal binding by the adenylyl cyclase toxin of *Bordetella pertussis*. *Arch. Biochem. Biophys*. **395,** 169–176.

Rogel, A. and Hanski, E. (1992). Distinct steps in the penetration of adenylate cyclase toxin of *Bordetella pertussis* into sheep erythrocytes. Translocation of the toxin across the membrane. *J. Biol. Chem*. **267,** 22599–22605.

Rogel, A., Meller, R. and Hanski, E. (1991). Adenylate cyclase toxin from *Bordetella pertussis*. The relationship between induction of cAMP and hemolysis. *J. Biol. Chem*. **266,** 3154–3161.

Rogel, A., Schultz, J.E., Brownlie, R.M., Coote, J.G., Parton, R. and Hanski, E. (1989). *Bordetella pertussis* adenylate cyclase: purification and characterization of the toxic form of the enzyme. *EMBO J.* **8,** 2755–2760.

Rogers, T.S., Corey, S.J. and Rosoff, P.M. (1990). Identification of a 43-kilodalton human T lymphocyte membrane protein as a receptor for pertussis toxin. *J. Immunol*. **145,** 678–683.

Roop, R.M.D., Veit, H.P., Sinsky, R.J., Viet, S.P., Hewlett, E.L. and Kornegay, E.T. (1987). Virulence factors of *Bordetella bronchiseptica* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. *Infect. Immun*. **55,** 217–222.

Rose, T., Sebo, P., Bellalou, J. and Ladant, D. (1995). Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin. Characterization of multiple calcium-binding sites and calcium-induced conformational changes. *J. Biol. Chem*. **270,** 26370–26376.

Sakamoto, H., Bellalou, J., Sebo, P. and Ladant, D. (1992). *Bordetella pertussis* adenylate cyclase toxin. Structural and functional independence of the catalytic and hemolytic activities. *J. Biol. Chem*. **267,** 13598–13602.

Saron, M.F., Fayolle, C., Sebo, P., Ladant, D., Ullmann, A. and Leclerc, C. (1997). Anti-viral protection conferred by recombinant adenylate cyclase toxins from *Bordetella pertussis* carrying a $CD8^+$ T cell epitope from lymphocytic choriomeningitis virus. *Proc. Natl. Acad. Sci. USA* **94,** 3314–3319.

Saukkonen, K., Burnette, W.N., Mar, V.L., Masure, H.R. and Tuomanen, E.I. (1992). Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proc. Natl. Acad. Sci. USA* **94,** 3314–3319.

Sato, H., Sato, Y. and Ohishi, I. (1991). Comparison of pertussis toxin (PT)-neutralizing activities and mouse protective activities of anti-PT mouse monoclonal antibodies. *Infect. Immun.* **59,** 3832–3835.

Scarlato, V., Arico, B., Prugnola, A. and Rappuoli, R. (1991). Sequential activation and environmental regulation of virulence genes in *Bordetella pertussis*. *EMBO J.* **10,** 3971–3975.

Schaeffer, L.M. and Weiss, A.A. (2001). Pertussis toxin and lipopolysaccharide influence phagocytosis of Bordetella pertussis by human monocytes. *Infect. Immun.* **69,** 7635–7641.

Scheuring, J. and Schramm, V.L. (1997a). Kinetic isotope effect characterization of the transition state for oxidized nicotinamide adenine nucleotide hydrolysis by pertussis toxin. *Biochemistry* **36,** 4526–4534.

Scheuring, J. and Schramm, V.L. (1997b). Pertussis toxin: transition state analysis for ADP-ribosylation of G-protein peptide alpha3C20. *Biochemistry* **36,** 8215–8223.

Schlecht, G., Loucka, J., Najar, H., Sebo, P. and Leclerc, C. (2004). Antigen targeting to CD11b allows efficient presentation of $CD4^+$ and $CD8^+$ T cell epitopes and *in vivo* Th1-polarized T cell priming. *J. Immunol*. **173,** 6089–6097.

Schmidt, G., Goehring, U.M., Schirmer, J., Lerm, M. and Aktories, K. (1999). Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for rho GTPases. *J. Biol. Chem.* **274,** 31875–31881.

Sebo, P., Glaser, P., Sakamoto, H. and Ullmann, A. (1991). High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system. *Gene* **104,** 19–24.

Sebo, P. and Ladant, D. (1993). Repeat sequences in the *Bordetella pertussis* adenylate cyclase toxin can be recognized as alternative carboxy-proximal secretion signals by the *Escherichia coli* alpha-hemolysin translocator. *Mol. Microbiol.* **9,** 999–1009.

Senda, T., Horiguchi, Y., Umemoto, M., Sugimoto, N. and Matsuda, M. (1997). *Bordetella bronchiseptica* dermonecrotizing toxin, which activates a small GTP-binding protein rho, induces membrane organelle proliferation and calveolae formation. *Exp. Cell Res.* **230,** 163–168.

Shen, Y., Lee, Y.S., Soelaiman, S., Bergson, P., Lu, D., Chen, A., Beckingham, K., Grabarek, Z., Mrksich, M. and Tang, W.J. (2002). Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. *EMBO J.* **21,** 6721–6732.

Shigeta, R. Jr. (1994). Isomorphous binding of mercury-substituted thiosaccharides to pertussis toxin crystals yields crystallographic phases. *Acta Crystallogr. D Biol. Crystallogr.* **50,** 71–74.

Shive, C.L., Hofstetter, H., Arredondo, L., Shaw, C. and Forsthuber, T.G. (2000). The enhanced antigen-specific production of cytokines induced by pertussis toxin is due to clonal expansion of T cells and not to altered effector functions of long-term memory cells. *Eur. J. Immunol.* **30,** 2422–2431.

Shumilla, J.A., Lacaille, V., Hornell, T.M., Huang, J., Narasimhan, S., Relman, D.A. and Mellins, E.D. (2004). *Bordetella pertussis* infection of primary human monocytes alters HLA-DR expression. *Infect. Immun.* **72,** 1450–1462.

Silvera, D., Edington, N. and Smith, I.M. (1982). Ultrastructural changes in the nasal turbinate bones of pigs in early infection with *Bordetella bronchiseptica*. *Res. Vet. Sci.* **33,** 37–42.

Simsova, M., Sebo, P. and Leclerc, C. (2004). The adenylate cyclase toxin from *Bordetella pertussis*—a novel promising vehicle for antigen delivery to dendritic cells. *Int. J. Med. Microbiol.* **293,** 571–576.

Smith, A.M., Yan, H., Groves, N., Dalla Pozza, T. and Walker, M.J. (2000). Co-expression of the *Bordetella pertussis* leader peptidase I results in enhanced processing and expression of the pertussis toxin S1 subunit in Escherichia coli. *FEMS Microbiol. Lett.* **191,** 177–182.

Sory, M.P. and Cornelis, G.R. (1994). Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. *Mol. Microbiol.* **14,** 583–594.

Stein, P.E., Boodhoo, A., Armstrong, G.D., Cockle, S.A., Klein, M.H. and Read, R.J. (1994a). The crystal structure of pertussis toxin. *Structure* **2,** 45–57.

Stein, P.E., Boodhoo, A., Armstrong, G.D., Heerze, L.D., Cockle, S.A., Klein, M.H. and Read, R.J. (1994b). Structure of a pertussis toxin-sugar complex as a model for receptor binding. *Nat. Struct. Biol.* **1,** 591–596.

Stenson, T.H. and Weiss, A.A. (2002). DsbA and DsbC are required for secretion of pertussis toxin by *Bordetella pertussis*. *Infect. Immun.* **70,** 2297–2303.

Su, S.B., Silver, P.B., Wang, P., Chan, C.C. and Caspi, R.R. (2003). Dissociating the enhancing and inhibitory effects of pertussis toxin on autoimmune disease. *J. Immunol.* **171,** 2314–2319.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry* **21,** 5516–5522.

Taranger, J., Trollfors, B., Bergfors, E., Knutsson, N., Lagergard, T., Schneerson, R. and Robbins, J.B. (2001). Immunologic and epidemiologic experience of vaccination with a monocomponent pertussis toxoid vaccine. *Pediatrics* **108,** E115.

Tonon, S., Goriely, S., Aksoy, E., Pradier, O., Del Giudice, G., Trannoy, E., Willems, F., Goldman, M. and De Wit, D. (2002). *Bordetella pertussis* toxin induces the release of inflammatory cytokines and dendritic cell activation in whole blood: impaired responses in human newborns. *Eur. J. Immunol.* **32,** 3118–3125.

Veithen, A., Raze, D. and Locht, C. (2000). Intracellular trafficking and membrane translocation of pertussis toxin into host cells. *Int. J. Med. Microbiol.* **290,** 409–413.

Vordermeier, H.M., Simsova, M., Wilkinson, K.A., Wilkinson, R.J., Hewinson, R.G., Sebo, P. and Leclerc, C. (2004). Recognition of mycobacterial antigens delivered by genetically detoxified *Bordetella pertussis* adenylate cyclase by T cells from cattle with bovine tuberculosis. *Infect. Immun.* **72,** 6255–6261.

Walker, K.E. and Weiss, A.A. (1994). Characterization of the dermonecrotic toxin in members of the genus *Bordetella. Infect. Immun.* **62,** 817–3828.

Weingart, C.L., Mobberley-Schuman, P.S., Hewlett, E.L., Gray, M.C. and Weiss, A.A. (2000). Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect. Immun.* **68,** 7152–7155.

Weingart, C.L. and Weiss, A.A. (2000). *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect. Immun.* **68,** 1735–1739.

Weiss, A.A. and Goodwin, M.S. (1989). Lethal infection by *Bordetella pertussis* mutants in the infant mouse model. *Infect. Immun.* **57,** 3757–3764.

Weiss, A.A., Hewlett, E.L., Myers, G.A. and Falkow, S. (1984). Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. *J. Infect. Dis.* **150,** 219–222.

Weiss, A.A., Johnson, F.D. and Burns, D.L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. *Proc. Natl. Acad. Sci. USA* **90,** 2970–2974.

Welch, R.A. (2001). RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr. Top. Microbiol. Immunol.* **257,** 85–111.

Wolff, J., Cook, G.H., Goldhammer, A.R. and Berkowitz, S.A. (1980). Calmodulin activates prokaryotic adenylate cyclase. *Proc. Natl. Acad. Sci. USA.* **77,** 3841–3844.

Xu, Y. and Barbieri, J.T. (1995). Pertussis toxin-mediated ADP-ribosylation of target proteins in Chinese hamster ovary cells involves a vesicle trafficking mechanism. *Infect. Immun.* **63,** 825–832.

Zaretzky, F.R., Gray, M.C. and Hewlett, E.L. (2002). Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin-filamentous hemagglutinin interaction. *Mol. Microbiol.* **45,** 1589–1598.

Zhou, G.C., Parikh, S.L., Tyler, P.C., Evans, G.B., Furneaux, R.H., Zubkova, O.V., Benjes, P.A. and Schramm, V.L. (2004). Inhibitors of ADP-ribosylating bacterial toxins based on oxacarbenium ion character at their transition states. *J. Am. Chem. Soc.* **126,** 5690–5698.

CHAPTER

# 17

# The Shiga toxins: properties and action on cells

*Kirsten Sandvig*

## INTRODUCTION

Shiga and Shiga-like toxins (Stxs) are produced by bacteria able to cause infectious disease (Karmali, 2004b; Karmali, 2004a; O'Loughlin and Robins-Browne, 2001). These protein toxins belong to the group of A-B toxins (Figure 17.1) (Lingwood, 1999; Sandvig, 2001; Sandvig and van Deurs, 2002; Lingwood, 2003). The binding part (B) of Shiga toxins consists of a pentamer that recognizes specific glycosphingolipids at the cell surface; most of these toxins bind to globotriasoylceramide (Gb3). After binding, the toxin is endocytosed and transported retrogradely to the Golgi apparatus and the endoplasmic reticulum (ER) (Sandvig *et al.*, 1992) from where the enzymatically active moiety (A or A1) is translocated to the cytosol (Figure 17.2). After entry into the cytosol, the toxin inactivates ribosomes by removing one adenine from the 28S RNA (Endo *et al.*, 1988), and thereby it inhibits protein synthesis. During entry into cells, the A-chain is cleaved by the cellular enzyme furin into the small A2 fragment and the enzymatically active part, the A1 fragment (Garred *et al.*, 1995b). The Shiga toxins can in some cells induce synthesis of cytokines (Foster and Tesh, 2002; Lee *et al.*, 2002), and the toxins are able to cause apoptosis (Yoshida *et al.*, 2002; Smith *et al.*, 2003). In most cells, apoptosis seems to be caused by ribotoxic stress (Smith *et al.*, 2003). Furthermore, binding of these toxins can induce signaling cascades that may contribute to the entry and the effects observed (Sandvig *et al.*, 2004).

Contaminated water and food are major sources of infection with bacteria producing Shiga toxins (Kumar *et al.*, 2004b; Karmali, 2004a). Although cattle and other animals (Urdahl *et al.*, 2004a) serve as a natural reservoir of Stx-producing *Escherichia coli* (STEC), not only beef but also contaminated seafood, vegetables, and fruits give rise to infections (Kumar *et al.*, 2004b; Karmali, 2004a; Kumar *et al.*, 2004a). Furthermore, also person-to-person transmission can easily occur since the infectious dose of *Escherichia coli* is very low (Karmali, 2004a). Infection with bacteria producing Shiga-like toxins is a threat to human health even in industrialized countries. Diarrhea caused by these bacteria can be followed by hemolytic uremic syndrome (HUS), which is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Karmali, 2004a). HUS can also be a complication to urinary tract infection by Shiga toxin producing *E. coli* (Karmali, 2004a). Especially children are at risk of getting kidney failure after such infections. Of infected persons, 5–10% will develop HUS, and 5% of these will die from the disease. The reader is referred to earlier reviews (Andreoli *et al.*, 2002; Siegler, 2003; Paton and Paton, 1998) when it comes to the development and characteristics of this disease where toxic effects are observed in the kidney endothelial cells as well as in other cell types. Studies so far reveal that although the Shiga toxins are related, their effects differ, and the outcome of an infection with bacteria producing one or more of these toxins is dependent on the type of toxin produced. Increased understanding of the mode of action of Shiga toxins is important both to

prevent and to cure toxin-induced disease (Karmali, 2004a; Karmali, 2004b). Importantly, Shiga toxins can also be used as tools in medicine and biology (Sandvig and van Deurs, 2002; Smith *et al.*, 2002b; Kim *et al.*, 1998; Facchini and Lingwood, 2001). This chapter will describe the various aspects of the Shiga toxins and their interactions with cells.

## THE SHIGA TOXINS AND THE BACTERIA THAT PRODUCE THEM

As shown in Figure 17.1, Shiga toxin, which is produced by *Shigella dysenteriae*, consists of a ring of five identical B-chains (the B-moiety), which is noncovalently associated with the A-chain of the toxin. The B-chains of Shiga toxin each have a molecular mass of 7,691, and each B-chain has been found to have three binding sites for Gb3. However, these are not equal in their ability to bind the receptor. As described below, the ability of one toxin molecule to bind multiple glycosphingolipid molecules is likely to be important not only for its strong binding to the cell surface but also for entry into the cells. The intact A-chain of Shiga toxin has a mass of 32,225, and importantly, it contains a loop formed by a disulfide bond involving the two cysteines in position 242 and 261. In this loop area, there is the sequence Arg-X-X-Arg that is the minimal recognizable motif for the cellular enzyme furin, which seems to be responsible for cleavage of the A-chain into the active A1-part and the inactive A2-peptide (Garred *et al.*, 1995b). In Shiga toxin, the active site of A1 is blocked by a methionine residue found in A2 (Fraser *et al.*, 1994). When cells without furin are transfected with this enzyme, they obtain the ability to cleave the toxin, and they do also become sensitized to Shiga toxin. Amino acid changes that involve removal of one of the arginines in the loop, or deletion of the loop, make the molecule resistant to cleavage by furin and reduce the effect of Shiga toxin on cells (Garred *et al.*, 1995a). Furin-induced cleavage of Shiga toxin occurs most efficiently at low pH (Garred *et al.*, 1995b), and since furin recycles between the cell surface and the endosomes, cleavage of the toxin can occur at an

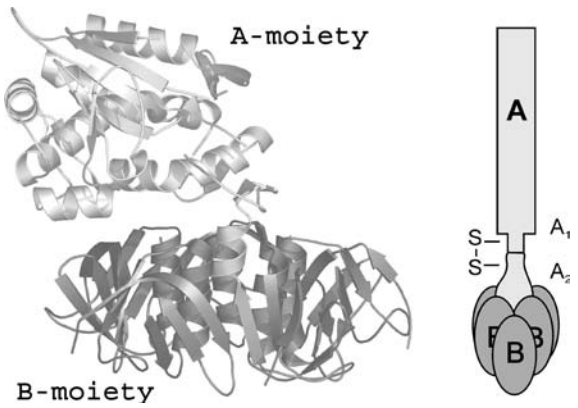



**FIGURE 17.1** Structure of Shiga toxin; chrystallographic and schematic (PDB protein data bank 1DMO). The binding moiety consists of the five small B fragment that are responsible for binding to glycosphingolipids (Gb3) at the cell surface. The A fragment can be cleaved by the cellular enzyme furin to A1 and A2, and the enzymatic activity resides in the A1 fragment.

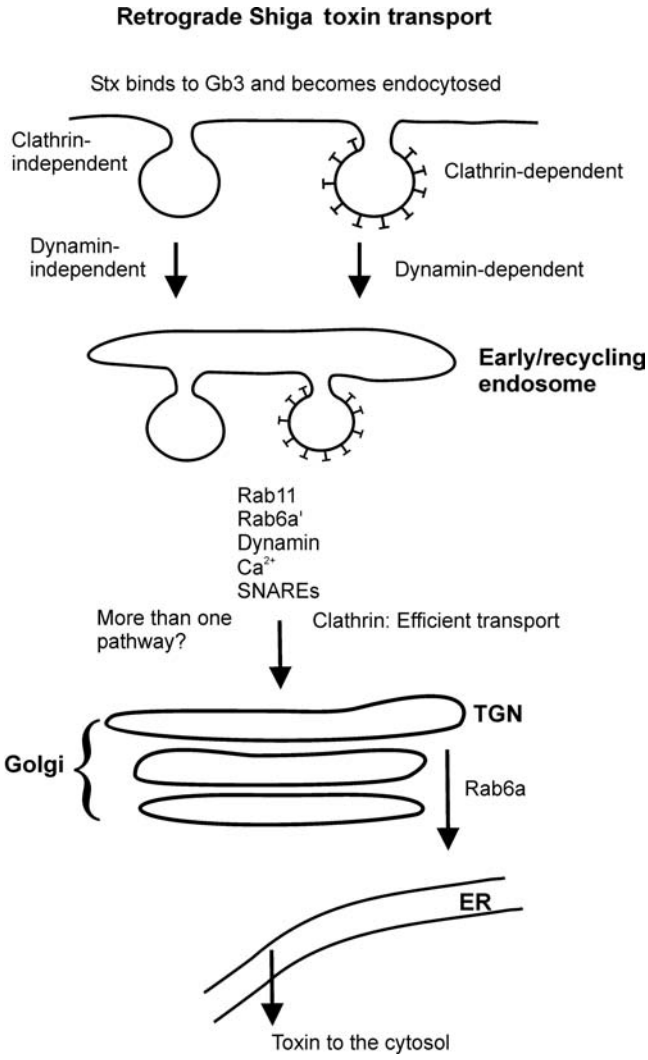



**FIGURE 17.2** Entry of Shiga toxin into cells. After endocytic uptake either from clathrin-coated pits or by clathrin-independent endocytosis, the toxin is transported to the Golgi apparatus by a process that can be dependent on clathrin, dynamin, and two different SNARE-complexes, as discussed in the text. After retrograde transport to the endoplasmic reticulum, at least the enzymatically active A1 fragment is transferred to the cytosol where it inactivates the ribosomes and thereby inhibits protein synthesis. This may then induce apoptosis (see main text).

optimal rate shortly after it is endocytosed. It should be noted that furin activates not only Shiga toxin but also other toxins such as diphtheria and anthrax toxin (Gordon and Leppla, 1994). However, the conditions for optimal cleavage differ and the location of toxin processing in the cell is therefore likely to be toxin-dependent. Thus, cleavage of anthrax occurs already at the cell surface (Klimpel *et al.*, 1992). After cleavage of Shiga toxin with furin, the internal disulfide bond in the A-chain will function to keep the two parts of the A-chain together and in association with the B-chain that can mediate retrograde transport of the enzymatically active part to the Golgi apparatus and the ER. If the disulfide is deleted by mutation of one of the cysteines (C242S), the A1-moiety of the A-chain is released from the rest of the molecule after cleavage with furin (Garred *et al.*, 1997). Even in cells without furin, there is a certain toxicity and cleavage of the A-chain. However, the toxicity is seen after longer times, and the cleavage occurs at a later stage of the intracellular journey of Shiga toxin since it (in contrast to furin-induced cleavage) is abolished by brefeldin A that disrupts the Golgi apparatus. Interestingly, inhibitors of the cytosolic enzyme calpain can reduce this processing, and one may therefore speculate as to whether the cleavage occurs after translocation of the intact A-chain to the cytosol (Garred *et al.*, 1995a). Different studies of Shiga toxin and Shiga-like toxins, as well as of the plant toxin ricin, reveal that there are two areas with sequence homology in the A-chains that are important for the enzymatic activity. For the A-chain of Shiga toxin, a change of glutamic acid in position 167 to aspartic acid was found to reduce the toxic effect of the molecule by a factor of 1,000 (Acheson and Keusch, 1999).

A number of Shiga-like toxins (Stxs) are produced by *Escherichia coli* and also other bacteria, such as *Aeromonas hydrophila, A. cavia, Citrobacter freundii*, and *Enterobacter cloacae,* have been reported to secrete similar toxins (Schmidt, 2001; Paton and Paton, 1998; Schmidt *et al.*, 1993). Furthermore, the fact that free Shiga toxin bacteriophages can be isolated from sewage (Muniesa *et al.*, 2004) might suggest that other bacteria can be toxin producers also. It should be noted that these toxins are also called *verotoxins* (VT) due to their strong toxic effect on Vero cells (names such as VT1 and VT2 are still commonly used). In contrast to Shiga toxin that is chromosomally encoded, the Stxs are encoded by prophages in *E. coli*, and they may therefore be transferred to other bacteria. Shiga-like toxin 1 (Stx1) is almost identical to Shiga toxin; there is only one amino acid that differs. However, although the differences are not large, some variants of Stx1 have been found and characterized (Paton *et al.*, 1995; Ohmura-Hoshino *et al.*, 2003; Suzuki *et al.*, 2004). In contrast, Shiga-like toxin 2 (Stx2) consists of several subgroups: Stx2, Stx2c (Schmitt *et al.*, 1991), 2d (Pierard *et al.*, 1998), 2e (Weinstein *et al.*, 1988), and 2f (Schmidt *et al.*, 2000). The toxins are not neutralized by antiserum to Shiga toxin, and the homology is around 60%. Interestingly, some bacteria produce more than one toxin variant. A number of different Shiga toxin types are produced in humans after infection. For instance, studies of 677 Shiga-toxin–producing *E. coli* revealed that they produced six different types of toxin (Stx1, Stx1c, Stx2, Stx2c, Stx2d, and Stx2e) (Beutin *et al.*, 2004). As mentioned, most of the Shiga-like toxins bind Gb3. A special case is Stx2e, secreted by bacteria that mostly infect pigs, since it binds to Gb4. Mutations of the B-chain affecting the binding specificity have been performed (Acheson and Keusch, 1999). Recent structural studies of Stx2 (Fraser *et al.*, 2004) reveal important differences between Stx and Stx2. For instance, in contrast to Stx (Fraser *et al.*, 1994), the active site of Stx2 is accessible in the crystal structure, the carboxyl termini of the A subunits of the two toxins differ in structure, and one of the receptor-binding domains in the B-pentamer differs in conformation. These differences are likely to be related to the fact that the toxins are far from identical in their interaction with cells and in development of disease.

## DETECTION OF SHIGA AND SHIGA-LIKE TOXINS

Detection of Stx-producing bacteria and of Stx can be performed by different methods, including enzyme-linked immunosorbent assays (ELISA), cytotoxicity assays, and polymerase chain reaction (PCR) (Pulz *et al.*, 2003; Shimada *et al.*, 1999; Kumar *et al.*, 2004a). Also, flow cytometry detection of Shiga toxins bound to polymorphonuclear leukocytes was reported to be a rapid, simple, and sensitive method to detect circulating Shiga toxin and to diagnose STEC infections (Tazzari *et al.*, 2004). For a historical overview of Stx discovery and purification, the reader is referred to Acheson and Keusch (Acheson and Keusch, 1999).

## BINDING OF TOXIN TO CELL SURFACE RECEPTORS

Binding of Stxs to cell surface receptors is a complex and, in general, a necessary first step for intoxication. However, binding to specific receptors does not always lead to a subsequent toxic effect (see below). As described above, the Stxs (with the exception of Stx 2e) bind to the sequence Gal α1-4 Gal-Glu of Gb3, but the

interaction with the carbohydrates is toxin-specific (Binnington *et al.*, 2002; Lingwood and Mylvaganam, 2003). This has been demonstrated by removal of hydroxyl-groups from the carbohydrates. Although mutational analysis of the Gb3-binding sites of the toxin B-chain suggests that all three binding sites of the B-chain play a role (Soltyk *et al.*, 2002), the interaction of the three sites with Gb3 is not identical. The role of one of these sites (site 3) in Stx 1B may be to orient the molecule at the cell surface. Not only the carbohydrate part of Gb3 but also the fatty acid in the receptor (Gb3) is important for binding (Binnington *et al.*, 2002). The length, the degree of unsaturation, and the hydroxylation of the fatty acid can determine the toxin binding. Importantly, the binding of the different Stxs are not affected to the same extent by a change in fatty acid composition, indicating that differences in effects on cells seen with various Stxs can be due to the interaction with the receptor and the location of Gb3 in the membrane. Gb3 with long saturated fatty acids is localized in lipid domains, in contrast to Gb3 with more unsaturated fatty acid, and intoxication and entry of Stxs into cells have been reported to be dependent on Gb3 composition and the presence of the receptor in rafts (Falguieres *et al.*, 2001; Kovbasnjuk *et al.*, 2001a). Not only the direct interaction of Stx with Gb3 is important for the binding. Interestingly, also the phospholipids in the surrounding membrane play a role (Arab and Lingwood, 1998; Arab and Lingwood, 1996), and the receptor-bound toxin itself has been reported to interact with surface proteins (Shimizu *et al.*, 2003). Such interactions could of course influence the location of the toxin-receptor complex, and the interactions could facilitate uptake of the toxin by specific endocytic pathways. Thus, although more Stx1 than Stx2 binds to human renal endothelial cells, these cells are more sensitive to Stx2 (Louise and Obrig, 1995). The possibility exists that Stx2 binds to receptors that are more prone to be internalized and transported to the right destination.

It should be mentioned that although glycolipids provide high-affinity binding sites for Stxs, the interactions with proteins at the cell surface of polymorphonuclear lymphocytes (PMNs) might play an important role in disease (te Loo *et al.*, 2000). Since this interaction is weaker than the one between Stx and glycolipids, these cells could function to bring the toxin from the site of infection to the kidney where the toxin would become associated with glycolipid receptors on kidney cells (te Loo *et al.*, 2000). The ability of kidney cells to bind Stx might be a function of age (Chark *et al.*, 2004) and could explain the larger susceptibility of children to get kidney failure. However, the interaction of Stx with different cell types seems to play a role in the development of disease. The recent finding that Stx binds to activated blood platelets (Ghosh *et al.*, 2004) may be important for the pathogenesis of HUS. However, the symptoms seen in connection with HUS can be caused by a combination of factors affecting cells. For instance, Shiga-like toxins might increase hemin-induced toxicity in renal tubular epithelial cells due to an inhibition of the cellular response to excess heme (Bitzan *et al.*, 2004).

The synthesis of Gb3 can, in certain cell types, be regulated by cytokines (for instance TNF-α and interleukin-1), by bacterial lipopolysaccharide (LPS), as well as by chemical substances such as butyric acid and increased levels of cAMP (Sandvig *et al.*, 2004; Lingwood, 1999). Since Stx in itself can induce synthesis of cytokines (see below), the toxin can to some extent regulate its own binding and effect on cells. The fact that quite high concentrations of butyric acid can be found in the intestine might be important for effects of Stx (McIntyre *et al.*, 1993; Fishman, 1982; Agarwal and Schimmel, 1989). It should be noted that the increased binding of Shiga toxin seen after treatment with butyric acid is not only due to an increased binding, it is also due to a change in the glycolipid composition of the cells facilitating transport of the toxin in the direction of the Golgi apparatus (Sandvig *et al.*, 1992; Sandvig *et al.*, 1996; Sandvig *et al.*, 1994). The induced production of Gb3 usually takes some time; a more rapid increase in exposure of Gb3 was recently reported to occur after treatment of endothelial cells with sphingomyelinase (Obrig *et al.*, 2003). Thus, a number of factors are important for the interaction of Stxs with surface receptors on cells.

Although action of Stx usually is dependent on interaction of the toxin with surface receptors, recent data suggest that the toxin might also have alternative ways to exert biological activity (Ferens *et al.*, 2004). Interestingly, the toxin was found to inhibit replication of bovine leukemia virus in a culture of bovine peripheral blood mononuclear cells, probably by entering directly through the permeabilized membrane of these cells (Ferens *et al.*, 2004).

## ENDOCYTIC UPTAKE OF SHIGA TOXIN

After binding of Shiga toxin to Gb3, the toxin-receptor complex is endocytosed. The uptake can occur by different endocytic pathways (Figure 17.2), and the fraction of toxin endocytosed by a given pathway is cell-type dependent and can be changed by the growth conditions (Lauvrak *et al.*, 2004; Khine *et al.*, 2004). In HeLa cells, the toxin can induce its own entry from clathrin-coated pits by a so far unknown mechanism

(Sandvig *et al.*, 1991; Sandvig *et al.*, 1989). Shiga toxin was the first lipid-binding toxin found to be internalized from clathrin-coated pits. More recently, it has been found that in some cells also cholera toxin can enter in a clathrin-dependent manner (Torgersen *et al.*, 2001; Shogomori and Futerman, 2001; Lencer and Tsai, 2003). It is possible that interactions with other surface molecules might play a role (Shimizu *et al.*, 2003). Along this line, it has been demonstrated that the glycosylphosphatidyl-anchored prion protein can enter from clathrin-coated pits due to an interaction of its protein part with other molecules at the external side of the membrane (Shyng *et al.*, 1995; Sunyach *et al.*, 2003).

As mentioned above, Stx can be internalized by different endocytic mechanisms (Nichols, 2002; Sandvig *et al.*, 2002; Lauvrak *et al.*, 2004; Lingwood, 1999; Khine *et al.*, 2004). Interestingly, when clathrin-dependent toxin uptake is blocked by expression of a dominant negative mutant of dynamin, which will inhibit uptake both from clathrin-coated pits and from caveolae, Stx is still endocytosed (Lauvrak *et al.*, 2004). Also when antisense to clathrin-heavy chain is expressed in BHK cells leading to an inhibition of clathrin function (Iversen *et al.*, 2003), Shiga toxin is taken in by endocytosis. In both cases, the uptake is reduced by about 50%. This does not necessarily reflect the fraction of toxin normally taken in by clathrin-independent endocytosis, since it has previously been shown that reduction of uptake from clathrin-coated pits by expression of mutant dynamin can up-regulate clathrin-independent endocytosis (Damke *et al.*, 1995). In some of the studies cited above, the uptake of Stx B-chain and not intact Stx was studied. Whether this makes a difference has not been reported, but could indeed be the case. Thus, at this point, one should be careful in making conclusions on the behavior of the intact toxin from studies of the B-chain. However, it is in agreement with studies of intact toxin (Lauvrak *et al.*, 2004) that similar investigations employing vector-based RNAi against clathrin heavy chain were shown to reduce also the rate of uptake of Stx-B chain (Saint-Pol *et al.*, 2004).

One can apparently regulate the fraction of toxin taken in by clathrin-dependent versus clathrin-independent endocytosis even in one and the same cell (Lauvrak *et al.*, 2004). When BHK cells are treated with butyric acid, they are sensitized to Stx, and a larger fraction seems to be internalized by clathrin-dependent endocytosis. As described below, clathrin also seems to play an important role for endosome to Golgi transport under these conditions. The Stx-receptor complex can be present in lipid rafts at the cell surface (Falguieres *et al.*, 2001). To what extent this is important for endocytosis of the toxin is not known, but one could imagine that it might affect the pathway used. As discussed below, raft association seems to be important for intracellular sorting of the toxin.

## TRANSPORT OF STX BETWEEN ENDOSOMES AND THE GOLGI APPARATUS

A critical step for intoxication with Stx is transport from endosomes to the Golgi apparatus (Figure 17.2) (Sannerud *et al.*, 2003; Sandvig and van Deurs, 2002). There are different lines of evidence indicating that Stx in sensitive cells is sorted directly from early and not via late endosomes to the Golgi apparatus (Mallard *et al.*, 1998; Sandvig *et al.*, 2002). Rab9-dependent transport from late endosomes to Golgi apparatus has been well characterized in the case of the mannose-6-phosphate receptor (Lombardi *et al.*, 1993; Riederer *et al.*, 1994), and we therefore produced cells with inducible synthesis of dominant negative mutant Rab 9 (Sandvig *et al.*, 2002). Upon induction of this mutant Rab9, there was a strong inhibition of Golgi transport of the mannose-6-phosphate receptor, whereas there was no effect on toxicity of Shiga toxin or on the Golgi transport of the related plant toxin ricin. Also, microscopical studies had already indicated that the Stx B-chain was transported from early endosomes to the Golgi, possibly via the perinuclear recycling compartment. In this compartment, the toxin could be seen in membrane areas coated with clathrin and containing AP1 (Mallard *et al.*, 1998). More recent studies have revealed a requirement for the clathrin adaptor epsin R, whereas AP1 does not seem to be required (Saint-Pol *et al.*, 2004). These results are in agreement with our data showing that clathrin is required for efficient endosome to Golgi transport (Lauvrak *et al.*, 2004). In cells sensitized to Stx with butyric acid, there is a strong inhibition of Stx transport to the Golgi apparatus upon induction of antisense to clathrin heavy chain. Interestingly, without sensitization to Stx by butyric acid, there is a smaller difference upon reduction of clathrin function and also a lower extent of protection against the toxin. These findings seem to reconcile some of the previous reports on the role/lack of role for clathrin in endosome to Golgi transport of Stx. In addition to clathrin, dynamin is important for this transport step (Lauvrak *et al.*, 2004), and it has been reported that also Rab11 and Rab6A′ are involved at this point (Sannerud *et al.*, 2003). Furthermore, SNAREs involved in this transport step (endosome to Golgi) have been characterized: the v-SNAREs VAMP3 and VAMP4 and the t-SNARES syntaxin 6, syntaxin 16, and Vti1A are important for this transport (Sannerud *et al.*, 2003; Johannes and Goud, 2000) and recently a second complex consisting of the

SNAREs syntaxin 5, GS28, Ykt6, and GS15 was found to be involved in endosome to Golgi transport of the StxB-chain (Tai *et al.*, 2004). When antibodies to these components were added to an *in vitro* system, the sulfation (a Golgi modification) of a modified StxB-chain was inhibited. The authors of this article suggest that there might be parallel pathways, employing different SNARE-complexes, operating between endosomes and the Golgi apparatus. Importantly, the level of cytosolic $Ca^{2+}$ plays a role in endosome to Golgi transport. Increasing levels of $Ca^{2+}$ will increase transport of Stx-B to Golgi by a factor of two in MDCK II cells (Lauvrak *et al.*, 2002), and it has been reported that decrease of the cytosolic $Ca^{2+}$ by a chelating agent that binds this ion decreased Shiga toxin transport to the Golgi (Chen *et al.*, 2002). However, in MDCK cells, increased levels of $Ca^{2+}$ have a much stronger effect on the transport of ricin (Lauvrak *et al.*, 2002) that presumably also is transported from early endosomes, supporting the idea that there could be multiple pathways or transport from different subdomains of endosomes. Interestingly, cholesterol is, as first shown for ricin (Grimmer *et al.*, 2000), also required for endosome to Golgi transport of Shiga toxin (Falguieres *et al.*, 2001). The exact role of cholesterol is, however, not known. Lipid rafts containing the toxin receptors may play a role in concentrating the receptors for transport in the direction of the Golgi apparatus, or such domains or cholesterol in itself could recruit cytosolic proteins of importance for vesicle formation or docking. Interesting in this context is the finding that glycolipids are not required for the retrograde transport of the plant toxin ricin (Spilsberg *et al.*, 2003).

## RETROGRADE STX TRANSPORT TO THE ER AND TRANSLOCATION OF THE A-CHAIN TO THE CYTOSOL

How does retrograde Stx transport occur? Stx does not have a KDEL sequence that could mediate retrograde transport by binding to the KDEL-receptor in COP I-coated vesicles (Calderwood *et al.*, 1987; Kozlov *et al.*, 1987). In agreement with this, saturation of KDEL receptors by lysozyme-KDEL expression did not protect cells against Stx1, whereas it did protect against *Pseudomonas* exotoxin A (Jackson *et al.*, 1999), which is believed to be transported retrogradely by exploiting the KDEL-system. However, different possibilities for retrograde transport exist, and Stx seems to use a Rab6A, COP I-independent route on its way to the ER and the cytosol (Figure 17.2) (White *et al.*, 1999; Girod *et al.*, 1999; Ghosh *et al.*, 2004). Studies of retrograde transport are complicated by the fact that the cells may use alternative pathways leading to the ER when one interferes with the normal routing. This was demonstrated to be the case in CHO cells where the Golgi was vesiculated by incubating cells with a temperature-sensitive ε-COP at the non-permissive temperature (Llorente *et al.*, 2003). At the non-permissive temperature the Golgi apparatus was vesiculated, and there seemed to be induction of a direct route between endosomes and the ER, a transport circumventing the Golgi vesicles. To investigate this compensatory routing, we used a modified form of the protein toxin ricin, a form that could be both sulfated by the sulfotransferase of the Golgi and glycosylated by ER-enzymes. Although Stx may be transported all the way to the ER in lipid rafts, it is not known whether the raft location is a requirement for TGN to ER transport (Ghosh *et al.*, 2004). Not surprisingly, actin may be important for retrograde transport (Luna *et al.*, 2002; Duran *et al.*, 2003). However, although actin seems to be required, too much actin and a strong network can actually inhibit the retrograde transport (Luna *et al.*, 2002; Duran *et al.*, 2003).

When Stx arrives in the ER, cells containing furin have already cleaved and activated the A-chain so it now consists of A1 connected to A2 by a disulfide bond (Garred *et al.*, 1995b). What happens to Stx is not known, but in analogy with cholera toxin that also has a disulfide bond which needs to be cleaved and where it has been shown that the ER-enzyme protein disulfide isomerase is involved in release of A1 from the rest of the molecule (Tsai *et al.*, 2001; Tsai and Rapoport, 2002), one might guess that this happens also in the case of Shiga toxin. However, this still has to be demonstrated. Also, it is not known how Stx is translocated from the ER to the cytosol, but again, in analogy with other toxins (Wesche *et al.*, 1999; Simpson *et al.*, 1999; Schmitz *et al.*, 2000), the Sec61-complex may be involved. This is the protein complex that is responsible for translocation of newly synthesized proteins from the cytosol to the ER, and this complex has been reported to be responsible for transfer of misfolded proteins from the ER to the cytosol where they are then subsequently degraded (Romisch, 1999). The Sec61-complex has, for instance, been found to interact with ricin (Wesche *et al.*, 1999; Simpson *et al.*, 1999) and with cholera toxin (Schmitz *et al.*, 2000). It should be noted that two recent publications (Ye *et al.*, 2004; Lilley and Ploegh, 2004) describe a new ER tranlocator, Derlin-1, responsible for transport of some proteins from the ER to the cytosol. Whether this protein might play a role in toxin transport remains to be investigated.

An important question that needs to be answered is: Which parts of Stx can enter the cytosol from the ER?

An enzymatically active part (presumably intact A1) is known to enter the cytosol since protein synthesis is inhibited, but can the whole A be translocated? The results obtained with Stx-mutants without the furin recognition site suggest that this is the case since these mutant molecules are cleaved by the cytosolic enzyme calpain (Garred *et al.*, 1995a). What about the B-chain? Could it also be translocated from the ER? It has been suggested that this is the case since epitopes orginally bound to the B-chain can be presented by MHC class I (Smith *et al.*, 2002b). However, there might be other explanation's for this presentation the epitope could for instance be transferred to the MHC class I in the ER (Smith *et al.*, 2002a). Also, it has been demonstrated that expression of the B-chain in the cytosol of cells leads to apoptosis (Nakagawa *et al.*, 1999), similarly as seen after addition of toxin to cells. However, these phenomena may be unrelated, and it is possible that B-chain production is toxic to cells. Thus, these questions still need clarification.

## TRANSPORT OF STX ACROSS EPITHELIAL CELLS

An important step in disease caused by Stx-producing bacteria is the transfer of the toxin across polarized epithelial cell layers. This can occur by transcytosis, a term that implies that the toxin is taken up into vesicles on one side of the cell layer and released again on the other side of the epithelial layer. Transcytosis of Stx1 and Stx2 has been demonstrated (Hurley *et al.*, 1999), and importantly the two toxins differ when it comes to how their transport is regulated. Disruption of microtubules with colchicine reduced the transport of Stx1 but not that of Stx2, whereas monensin (an ionophore that will increase the pH of acidic endosomes) decreased transport of both of them. Surprisingly, addition of Stx2 did not block transport of Stx1 (Hurley *et al.*, 1999). Whether differences between these two toxins at this stage contribute to the fact that Stx2 seems to be more potent in causing disease is not known. In polarized Caco-2 cells, the internalization of the Stx1 B-subunit was found to be dependent on lipid rafts (Kovbasnjuk *et al.*, 2001b). However, whether this is a general phenomenon for polarized epithelial cells and whether the same applies to Stx2 has not yet been investigated.

The toxins might also cross the epithelial cell layer by a paracellular route. Studies of the epithelial cell line T84 revealed that Stx-producing bacteria could induce synthesis of interleukin 8 and transmigration of polymorphonuclear leukocytes (Hurley *et al.*, 2001). This movement of leukocytes increased paracellular permeability to Stx (Hurley *et al.*, 2001), and would thereby increase the chance of HUS development. Another cell type that might play a role in disease caused by Stx-producing bacteria is the macrophage. If the epithelial cell barrier is disrupted, then macrophages can engulf the bacteria. Importantly, even when the bacteria are taken up and killed by the macrophages, these cells secrete toxin to the surroundings (Shimada *et al.*, 1999).

## INDUCTION OF CYTOKINE PRODUCTION

Stxs are able to induce production of cytokines such as interleukin 1, 6, and 8 after interacting with different types of cells (Cameron *et al.*, 2003; Smith *et al.*, 2003; Lee *et al.*, 2002; Thorpe *et al.*, 2001; Zoja *et al.*, 2002; Matussek *et al.*, 2003). In most cases, production of the interleukins and other peptides seems to be due to the stress caused by toxin-induced inactivation of the ribosomes (Thorpe *et al.*, 1999; Yamasaki *et al.*, 1999). This idea is supported by the following results: The protein toxin ricin has a similar effect as Stx on the intestinal epithelial cell HCT-8; both toxins induce secretion of interleukin-8 (Thorpe *et al.*, 1999), suggesting that the response is not due to the ability of Stx to selectively interact with Gb3. Also, a non-toxic mutant of Stx does not induce cytokine production (Yamasaki *et al.*, 1999). Interestingly, chemokine mRNAs can both be induced and stabilized by addition of Stx to cells (Thorpe *et al.*, 2001).

Toxin-induced activation of various protein kinases seems crucial for the production and release of cytokines. For instance, inhibition of MAP kinase (mitogen activated protein kinase) p38 resulted in reduced release of interleukin 8 (Thorpe *et al.*, 1999). Furthermore, there is evidence for involvement of other MAP kinases, such as JNK (Smith *et al.*, 2003). In monocytes, p38 and the kinase ERK seem to be involved in Stx-induced production of tumor necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulated factor (GM-CSF) (Cameron *et al.*, 2003; Foster and Tesh, 2002). Quite recently, it was reported that Stx1 could induce release of adenine by a direct effect on DNA (Brigotti *et al.*, 2002), suggesting that the toxin can use different strategies to attack cells. Thus, the possibility exists that the ability of the toxin to change transcription could be related to the direct effect on nuclear DNA (Brigotti *et al.*, 2002).

There is evidence that the toxin-induced release of various active polypeptides is important for the development of disease after infection with Stx-producing bacteria. For instance, in response to Stx-induced

release of chemokines, neutrophiles may be recruited to the gut (the site of infection), and the intestinal barrier could become affected (Thorpe *et al.*, 2001). As discussed already, an important effect of some of the released active proteins is that they can induce synthesis of Gb3, the receptor for Stx, on other cell types and thereby make them sensitive to the toxin (Sandvig *et al.*, 2004).

The finding that Stx2 seems to be more potent than Stx1 in inducing HUS could be related to differences in the ability of these toxins to regulate cytokine production (Siegler *et al.*, 2003; Matussek *et al.*, 2003). Importantly, studies with cDNA arrays revealed that Stx2 produced by *E. coli* O157:7 isolated from a patient with HUS had the ability to up-regulate one-third of cytokine genes in renal tubular cells (Lee *et al.*, 2002), and it was suggested that these changes are associated with the renal injury seen in HUS. Studies in baboons (Siegler *et al.*, 2003) have actually demonstrated a difference in cytokine response after administration of Stx2 and Stx1. In that model, some of the changes associated with HUS were only observed with Stx2.

## TOXIN-INDUCED APOPTOSIS

Shiga toxins are known to induce apoptosis in several cell types, but the mechanisms involved seem to be cell-type dependent (Cherla *et al.*, 2003). At least in some cell types, the apoptosis mediated by Stx is induced after entry of the A-chain into the cytosol since disruption of the Golgi apparatus by the drug brefeldin A protects the cells not only against inhibition of protein synthesis but also against Stx-induced apoptosis (Yoshida *et al.*, 2002; Kojio *et al.*, 2000; Fujii *et al.*, 2003). Some of the stress-induced kinases, such as JNK and the MAP kinase p38, are reported to be involved in apoptosis since an inhibition of these kinases protected cells against this process (Smith *et al.*, 2003). Furthermore, Stx-induced apoptosis can be mediated by activation of caspases (Tetaud *et al.*, 2003; Smith *et al.*, 2003; Cherla *et al.*, 2003), but also caspase-independent, toxin-induced apoptosis has been reported (Cherla *et al.*, 2003). Inhibition of synthesis of the antiapoptotic protein MCl-1 (a Bcl-2 family member) may be involved in Stx-induced apoptosis in human dermal microvascular endothelial cells, and inhibition of protein degradation in these cells by addition of lactacystin prevented the toxin-induced apoptosis (Erwert *et al.*, 2003). Furthermore, overexpression of Bcl-2 in Hep-2 cells protected against apoptosis caused by Stx1 and Stx2 (Jones *et al.*, 2000). How the toxin-induced inactivation of ribosomes leads to activation of kinases and caspases is not known.

Cells can also undergo apoptosis in response to the B-chain only (Mori *et al.*, 2000; Tetaud *et al.*, 2003), suggesting that in some cases signaling caused by the receptor-bound B moiety is sufficient to induce apoptosis. A rapid activation of the tyrosine kinase Syk and the Src family kinase Lyn was observed in Ramos cells (Mori *et al.*, 2000). Interstingly, Stx and antibodies to Gb3 both induce apoptosis in Burkitt's lymphoma cells (Tetaud *et al.*, 2003), but different mechanisms are involved: Only the toxin-induced process was dependent on caspases (Tetaud *et al.*, 2003). Also, in the human renal tubular endothelial cells (ACHN), Stx rapidly activated the Scr kinase family member Yes (Katagiri *et al.*, 1999). Interestingly, in ACHN cells the Stx B subunit is sufficient to induce a cytoskeletal remodeling, which is dependent on Src kinases and Rho-associated kinase (Takenouchi *et al.*, 2004). A redistribution of a number of proteins including ezrin, CD44, vimentin, actin, and tubulin was demonstrated (Takenouchi *et al.*, 2004). However, the consequences of these changes need to be clarified. During disease caused by Stx-secreting bacteria, multiple factors are likely to contibute. Importantly, an apoptotic response can be increased by simultaneous exposure of cells to Stx, LPS (lipopolysacharide), and cytokines (Cherla *et al.*, 2003; Pijpers *et al.*, 2001).

## PROTECTION AGAINST SHIGA TOXINS

Both Shiga toxin produced by *Shigella dysenteriae* and Stxs produced by other bacteria can get into the bloodstream of the infected person and cause HUS. The Stxs also cause hemorrhagic colitis, although the role for Shiga toxin in Shigellosis is unclear (Acheson and Keusch, 1999). In contrast to Stx-secreting *E. coli*, *Shigella dysenteriae* invades the epithelial cells of the gut. Protection against the toxin by neutralizing it in the intestine and/or after transport to the blood is attempted in various ways. Immunization has been tried for some time (Acheson and Keusch, 1999), and more recently Stx-liposome conjugates are being investigated for effectiveness in vaccination (Uchida, 2003). New types of therapies are developed based on the detailed knowledge about the toxin-receptor interactions. Soluble receptor-based compounds able to bind Stxs after passage into the circulation or in the gut have been designed (Mulvey *et al.*, 2003; Karmali, 2004b; Watanabe *et al.*, 2004). Slight modification of these compounds can even change the specificity when it comes

to binding the different toxins (Mulvey *et al.*, 2003). Another approach to protect against the Stxs is to use bacteria that have been engineered to express Stx-binding molecules at the surface, thereby making these bacteria able to bind Stx and prevent spread to the circulation (Paton *et al.*, 2000). Interestingly, naturally occurring *E. coli* can produce LPS able to neutralize Stx2, and such bacteria might be used as probiotics (Gamage *et al.*, 2004). Improved technology and increased knowledge about the action of Stx are providing us with new tools to prevent and treat disease caused by toxin-producing bacteria.

## EXPLOITATION OF SHIGA TOXIN IN MEDICINE

Several protein toxins are used in construction of hybrid molecules (including immunotoxins), where a foreign binding part directs the hybrid molecule to the cell one wants to kill. For instance, both diphteria toxin and ricin conjugates directed against cancer cells have been prepared (Frankel *et al.*, 2002; Frankel *et al.*, 2000; Kreitman, 2003; Sinha, 2003; Thorburn *et al.*, 2004). In the case of Stxs, a similar approach might be used. However, it has also been suggested that Stx can be used against some forms of cancer without any modification (Siegler *et al.*, 2003; Arab *et al.*, 1999; Salhia *et al.*, 2002). Also, non-toxic derivatives of Stx were recently shown to possess adjuvant activity for mucosal immunity (Ohmura-Hoshino *et al.*, 2004).

Importantly, protein toxins can be used to obtain presentation of epitopes by MHC class I (Moron *et al.*, 2004; Smith *et al.*, 2002b). Parts of the toxin or inactivate mutant forms of the toxin with an extension containing the epitope to be presented are then incubated with cells, and the epitope is released from the modified toxin and presented by MHC class I (Moron *et al.*, 2004; Smith *et al.*, 2002b). In the case of Stx, epitopes have been added both to the A- and to the B-chain, and they are subsequently presented by MHC class I (Lee *et al.*, 1998; Haicheur *et al.*, 2000; Noakes *et al.*, 1999). Another possibility would be to add proteins with a biological function either to the A- or B-chain in order to obtain transport to the cytosol, as has been observed for other protein toxins (Wiedlocha *et al.*, 1994; Aullo *et al.*, 1993).

## CONCLUSION

The number of Shiga toxins that are discovered and characterized has been increasing, and our knowledge about the action of these toxins on single cells as well as in the whole organism is expanding. However, future research is required to obtain a complete understanding of the complex mechanism of action of these toxins and to develop new strategies to prevent and cure disease caused by the members of the Shiga toxin family. The exploitation of these fascinating toxins as vehicles in medicine, as well as probes to study general questions in cell biology, can be expected to lead to new discoveries.

## ACKNOWLEDGMENTS

Assistance with figures was kindly provided by Anne Grethe Myrann. The author is grateful to Drs. Tore-Geir Iversen and Sébastien Wälchli, members of the Sandvig-laboratory, for critical reading of the manuscript. The author has been supported by the Norwegian Cancer Society, the Norwegian Research Council for Science and the Humanities, the Novo Nordisk Foundation, the Jahre Foundation, and Jeanette and Søren Bothners Legacy.

## REFERENCES

Acheson, D.W.K. and G.T. Keusch. (1999). The family of Shiga toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins, Second Edition* (eds. J. E. Alouf and J. H. Freer), pp. 229–242. Academic Press, London.

Agarwal, V.P. and Schimmel, E.M. (1989). Diversion colitis: A nutritional deficiency syndrome? *Nutr. Rev.* **47,** 257–261.

Andreoli, S.P., Trachtman, H., Acheson, D.W.K., Siegler, R.L. and Obring, T.G. (2002). Hemolytic uremic syndrome: epidemiology, pathophysiology, and therapy. *Pediatr. Nephrol.* **17,** 293–298.

Arab, S. and Lingwood, C.A. (1996). Influence of phospholipid chain length on verotoxin/globotriaosyl ceramide binding in model membranes: comparison of a supported bilayer film and liposomes. *Glycoconj. J.* **13,** 159–166.

Arab, S. and Lingwood, C.A. (1998). Intracellular targeting of the endoplasmic reticulum/nuclear envelope by retrograde transport may determine cell hypersensitivity to verotoxin via globotriasyl ceramide fatty acid isoform traffic. *J. Cell. Physiol.* **177,** 646–660.

Arab, S., Rutka, J. and Lingwood, C. (1999). Verotoxin induces apoptosis and the complete, rapid, long-term elimination of human astrocytoma xenografts in nude mice. *Oncol. Res.* **11,** 33–39.

Aullo, P., Giry, M., Olsnes, S., Popoff, M.R., Kocks, C. and Boquet, P. (1993). A chimeric toxin to study the role of the 21 kDa GTP binding protein rho in the control of actin microfilament assembly. *EMBO J.* **12,** 921–931.

Beutin, L., Krause, G., Zimmermann, S., Kaulfuss, S. and Gleier, K. (2004). Characterization of Shiga toxin producing *Escherichia coli* strains isolated from human patients in Germany over a three-year period. *J. Clin. Microbiol.* **42,** 1099–1108.

Binnington, B., Lingwood, D., Nutikka, A. and Lingwood, C.A. (2002). Effect of globotriaosyl ceramide fatty acid alpha-hydroxylation on the binding by verotoxin 1 and verotoxin 2. *Neurochem. Res.* **27,** 807–813.

Bitzan, M., Bickford, B.B. and Foster, G.H. (2004). Verotoxin (shiga toxin) sensitizes renal epithelial cells to increased heme toxicity: possible implications for the hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* **15,** 2334–2343.

Brigotti, M., Alfieri, R., Sestili, P., Bonelli, M., Petronini, P.G., Guidarelli, A., Barbieri, L., Stirpe, F. and Sperti, S. (2002). Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. *FASEB J.* **16,** 365–372.

Calderwood, S.B., AuClair, F., Donohue-Rolfe, A., Keusch, G.T. and Mekalanos, J.J. (1987). Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **84,** 4364–4368.

Cameron, P., Smith, S.J., Giembycz, M.A., Rotondo, D. and Plevin, R. (2003). Verotoxin activates mitogen-activated protein kinase in human peripheral blood monocytes: role in apoptosis and proinflammatory cytokine release. *Br. J. Pharmacol.* **140,** 1320–1330.

Chark, D., Nutikka, A., Trusevych, N., Kuzmina, J. and Lingwood, C. (2004). Differential carbohydrate epitope recognition of globotriaosyl ceramide by verotoxins and a monoclonal antibody. *Eur. J. Biochem.* **271,** 405–417.

Chen, J.L., Ahluwalia, J.P. and Stamnes, M. (2002). Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. *J. Biol. Chem.* **277,** 35682–35687.

Cherla, R.P., Lee, S.Y. and Tesh, V.L. (2003). Shiga toxins and apoptosis. *FEMS Microbiol. Lett.* **228,** 159–166.

Damke, H., Baba, T., van der Bliek, A.M. and Schmid, S.L. (1995). Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell Biol.* **131,** 69–80.

Duran, J.M., Valderrama, F., Castel, S., Magdalena, J., Tomas, M., Hosoya, H., Renau-Piqueras, J., Malhotra, V. and Egea, G. (2003). Myosin motors and not actin comets are mediators of the actin-based golgi-to-endoplasmic reticulum protein transport. *Mol. Biol. Cell* **14,** 445–459.

Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988). Site of action of Vero toxin (VT2) from *Escherichia coli* 0157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA glycosidase activity of the toxins. *Eur. J. Biochem.* **171,** 45–50.

Erwert, R.D., Eiting, K.T., Tupper, J.C., Winn, R.K., Harlan, J.M. and Bannerman, D.D. (2003). Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. *Microb. Pathog.* **35,** 87–93.

Facchini, L.M. and Lingwood, C.A. (2001). A verotoxin 1 B subunit-lambda CRO chimeric protein specifically binds both DNA and globotriaosylceramide (Gb(3)) to effect nuclear targeting of exogenous DNA in Gb(3) positive cells. *Exp. Cell Res.* **269,** 117–129.

Falguieres, T., Mallard, F., Baron, C., Hanau, D., Lingwood, C., Goud, B., Salamero, J. and Johannes, L. (2001). Targeting of shiga toxin b-subunit to retrograde transport route in association with detergent-resistant membranes. *Mol. Biol. Cell* **12,** 2453–2468.

Ferens, W.A., Grauke, L.J. and Hovde, C.J. (2004). Shiga toxin 1 targets bovine leukemia virus-expressing cells. *Infect. Immun.* **72,** 1837–1840.

Fishman, P.H. (1982). Role of membrane gangliosides in the binding and action of bacterial toxins. *J. Membr. Biol.* **69,** 85–97.

Foster, G.H. and Tesh, V.L. (2002). Shiga toxin 1-induced activation of c-Jun NH(2)-terminal kinase and p38 in the human monocytic cell line THP-1: possible involvement in the production of TNF-alpha. *J. Leukoc. Biol.* **71,** 107–114.

Frankel, A.E., Kreitman, R.J. and Sausville, E.A. (2000). Targeted toxins. *Clin. Cancer Res.* **6,** 326–334.

Frankel, A.E., Powell, B.L. and Lilly, M.B. (2002). Diphtheria toxin conjugate therapy of cancer. In: *Cancer Chemotherapy and Biological Response Modifiers* (eds. G. Giaccone, R. Shcilsky, and P. Sondel), pp. 301–312. Elsevier Science, B.V.

Fraser, M.E., Chernaia, M.M., Kozlov, Y.V. and James, M.N. (1994). Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 A resolution. *Nat. Struct. Biol.* **1,** 59–64.

Fraser, M.E., Fujinaga, M., Cherney, M.M., Melton-Celsa, A.R., Twiddy, E.M., O'Brien, A.D. and James, M.N. (2004). Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J. Biol. Chem.* **279,** 27511–27517.

Fujii, J., Matsui, T., Heatherly, D.P., Schlegel, K.H., Lobo, P.I., Yutsudo, T., Ciraolo, G.M., Morris, R.E. and Obrig, T. (2003). Rapid apoptosis induced by Shiga toxin in HeLa cells. *Infect. Immun.* **71,** 2724–2735.

Gamage, S.D., McGannon, C.M. and Weiss, A.A. (2004). *Escherichia coli* serogroup O107/O117 lipopolysaccharide binds and neutralizes Shiga toxin 2. *J. Bacteriol.* **186,** 5506–5512.

Garred, Ø., Dubinina, E., Holm, P.K., Olsnes, S., van Deurs, B., Kozlov, J.V. and Sandvig, K. (1995a). Role of processing and intracellular transport for optimal toxicity of Shiga toxin and toxin mutants. *Exp. Cell Res.* **218,** 39–49.

Garred, Ø., Dubinina, E., Polesskaya, A., Olsnes, S., Kozlov, J. and Sandvig, K. (1997). Role of the disulfide bond in Shiga toxin A-chain for toxin entry into cells. *J. Biol. Chem.* **272,** 11414–11419.

Garred, Ø., van Deurs, B. and Sandvig, K. (1995b). Furin-induced cleavage and activation of Shiga toxin. *J. Biol. Chem.* **270,** 10817–10821.

Ghosh, S.A., Polanowska-Grabowska, R.K., Fujii, J., Obrig, T. and Gear, A.R. (2004). Shiga toxin binds to activated platelets. *J. Thromb. Haemost.* **2,** 499–506.

Girod, A., Storrie, B., Simpson, J.C., Johannes, L., Goud, B., Roberts, L.M., Lord, J.M., Nilsson, T. and Pepperkok, R. (1999). Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nature Cell Biol.* **1,** 423–430.

Gordon, V.M. and Leppla, S.H. (1994). Proteolytic activation of bacterial toxins: Role of bacterial and host cell proteases. *Infect. Immun.* **62,** 333–340.

Grimmer, S., Iversen, T.G., van Deurs, B. and Sandvig, K. (2000). Endosome to Golgi transport of ricin is regulated by cholesterol. *Mol. Biol. Cell* **11,** 4205–4216.

Haicheur, N., Bismuth, E., Bosset, S., Adotevi, O., Warnier, G., Lacabanne, V., Regnault, A., Desaymard, C., Amigorena, S., Ricciardi-Castagnoli, P., Goud, B., Fridman, W.H., Johannes, L. and Tartour, E. (2000). The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens. *J. Immunol.* **165,** 3301–3308.

Hurley, B.P., Jacewicz, M., Thorpe, C.M., Lincicome, L.L., King, A.J., Keusch, G.T. and Acheson, D.W. (1999). Shiga toxins 1 and 2 translocate differently across polarized intestinal epithelial cells. *Infect. Immun.* **67,** 6670–6677.

Hurley, B.P., Thorpe, C.M. and Acheson, D.W. (2001). Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. *Infect. Immun.* **69,** 6148–6155.

Iversen, T.-G., Skretting, G., van Deurs, B. and Sandvig, K. (2003). Formation of clathrin-coated pits with long dynamin-wrapped necks upon inducible expression of antisense to clathrin. *Proc. Natl. Acad. Sci.* USA **100,** 5175–5180.

Jackson, M.E., Simpson, J.C., Girod, A., Pepperkok, R., Roberts, L.M. and Lord, J.M. (1999). The KDEL retrieval system is exploited by *Pseudomonas* exotoxin A, but not by Shiga-like toxin-1, during retrograde transport from the Golgi complex to the endoplasmic reticulum. *J. Cell Sci.* **112,** 467–475.

Johannes, L. and Goud, B. (2000). Facing inward from compartment shores: How many pathways were we looking for? *Traffic* **1,** 119–123.

Jones, N.L., Islur, A., Haq, R., Mascarenhas, M., Karmali, M.A., Perdue, M.H., Zanke, B.W. and Sherman, P.M. (2000). *Escherichia*

*coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. *Am. J. Physiol. Gastrointest. Liver Physiol*. **278,** G811–G819.

Karmali, M.A. (2004a). Infection by Shiga toxin-producing *Escherichia coli*: an overview. *Mol. Biotechnol*. **26,** 117–122.

Karmali, M.A. (2004b). Prospects for preventing serious systemic toxemic complications of Shiga toxin-producing *Escherichia coli* Infections using Shiga toxin receptor analogues. *J. Infect. Dis.* **189,** 355–359.

Katagiri, Y.U., Mori, T., Nakajima, H., Katagiri, C., Taguchi, T., Takeda, T., Kiyokawa, N. and Fujimoto, J. (1999). Activation of Src family kinase yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. *J. Biol. Chem*. **274,** 35278–35282.

Khine, A.A., Tam, P., Nutikka, A. and Lingwood, C.A. (2004). Brefeldin A and filipin distinguish two globotriaosyl ceramide/verotoxin-1 intracellular trafficking pathways involved in Vero cell cytotoxicity. *Glycobiology* **14,** 701–712.

Kim, J.H., Johannes, L., Goud, B., Antony, C., Lingwood, C.A., Daneman, R. and Grinstein, S. (1998). Noninvasive measurement of the pH of the endoplasmic reticulum at rest and during calcium release. *Proc. Natl. Acad. Sci. USA*. **95,** 2997–3002.

Klimpel, K.R., Molloy, S.S., Thomas, G. and Leppla, S.H. (1992). Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. USA* **89,** 10277–10281.

Kojio, S., Zhang, H., Ohmura, M., Gondaira, F., Kobayashi, N. and Yamamoto, T. (2000). Caspase-3 activation and apoptosis induction coupled with the retrograde transport of Shiga toxin: inhibition by brefeldin A. *FEMS Immunol. Med. Microbiol*. **29,** 275–281.

Kovbasnjuk, O., Edidin, M. and Donowitz, M. (2001a). Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J. Cell Sci*. **114,** 4025–4031.

Kovbasnjuk, O., Edidin, M. and Donowitz, M. (2001b). Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J. Cell Sci*. **114,** 4025–4031.

Kozlov, Y.V., Kabishev, A.A., Fedchenko, V.I. and Bayev, A.A. (1987). Cloning and sequencing of Shiga toxin structural genes. *Proc. Natl. Acad. Sci. USSR* **295,** 740–744.

Kreitman, R.J. (2003). Taming ricin toxin. *Nat. Biotechnol*. **21,** 372–374.

Kumar, H.S., Karunasagar, I., Karunasagar, I., Teizou, T., Shima, K. and Yamasaki, S. (2004a). Characterization of Shiga toxin-producing *Escherichia coli* (STEC) isolated from seafood and beef. *FEMS Microbiol. Lett*. **233,** 173–178.

Kumar, H.S., Karunasagar, I., Karunasagar, I., Teizou, T., Shima, K. and Yamasaki, S. (2004b). Characterization of Shiga toxin-producing *Escherichia coli* (STEC) isolated from seafood and beef. *FEMS Microbiol. Lett*. **233,** 173–178.

Lauvrak, S.U., Llorente, A., Iversen, T.-G. and Sandvig, K. (2002). Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. *J. Cell Sci*. **115,** 3449–3456.

Lauvrak, S.U., Torgersen, M.L. and Sandvig, K. (2004). Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. *J. Cell Sci*. **117,** 2321–2331.

Lee, J.E., Kim, J.S., Choi, I.H., Tagawa, M., Kohsaka, T. and Jin, D.K. (2002). Cytokine expression in the renal tubular epithelial cells stimulated by Shiga toxin 2 of *Escherichia coli* O157:H7. *Ren Fail*. **24,** 567–575.

Lee, R.-S., Tartour, E., van der Bruggen, P., Vantomme, V., Joyeux, I., Goud, B., Fridman, W.H. and Johannes, L. (1998). Major histocompatibility complex class I presentation of exogenous tumor antigen fused to the B-fragment of Shiga toxin. *Eur. J. Immunol*. **28,** 2726–2737.

Lencer, W.I. and Tsai, B. (2003). The intracellular voyage of cholera toxin: going retro. *Trends Biochem. Sci*. **28,** 639–645.

Lilley, B.N. and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429,** 834–840.

Lingwood, C.A. (1999). Glycolipid receptors for verotoxin and *Helicobacter pylori:* role in pathology. *Biochim. Biophys. Acta* **1455,** 375–386.

Lingwood, C.A. (2003). Shiga toxin receptor glycolipid binding. Pathology and utility. *Methods Mol. Med*. **73,** 165–186.

Lingwood, C.A. and Mylvaganam, M. (2003). Lipid modulation of glycosphingolipid (GSL) receptors: soluble GSL mimics provide new probes of GSL receptor function. *Methods Enzymol*. **363,** 264–283.

Llorente, A., Lauvrak, S.U., van Deurs, B. and Sandvig, K. (2003). Induction of direct endosome to endoplasmic reticulum transport in Chinese hamster ovary (CHO) cells (LdlF) with a temperature-sensitive defect in epsilon-coatomer protein (epsilon-COP). *J. Biol. Chem*. **278,** 35850–35855.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M. and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J.* **12,** 677–682.

Louise, C.B. and Obrig, T.G. (1995). Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. *J. Infect. Dis*. **172,** 1397–1401.

Luna, A., Matas, O.B., J.A., Mato, E., Duran, J.M., Ballesta, J., Way, M. and Egea, G. (2002). Regulation of protein transport from the Golgi complex to the endoplasmic reticulum by CDC42 and N-WASP. *Mol. Biol. Cell* **13,** 866–879.

Mallard, F., Antony, C., Tenza, D., Salamero, J., Goud, B. and Johannes, L. (1998). Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin B-fragment transport. *J. Cell Biol*. **143,** 973–990.

Matussek, A., Lauber, J., Bergau, A., Hansen, W., Rohde, M., Dittmar, K.E., Gunzer, M., Mengel, M., Gatzlaff, P., Hartmann, M., Buer, J. and Gunzer, F. (2003). Molecular and functional analysis of Shiga toxin-induced response patterns in human vascular endothelial cells. *Blood* **102,** 1323–1332.

McIntyre, A., Gibson, P.R. and Young, G.P. (1993). Butyrate production from dietary fiber and protection against bowel cancer in a rat model. *Gut* **34,** 386–391.

Mori, T., Kiyokawa, N., Katagiri, Y.U., Taguchi, T., Suzuki, T., Sekino, T., Sato, N., Ohmi, K., Nakajima, H., Takeda, T. and Fujimoto, J. (2000). Globotriaosyl ceramide (CD77/Gb3) in the glycolipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. *Exp. Hematol*. **28,** 1260–1268.

Moron, G., Dadaglio, G. and Leclerc, C. (2004). New tools for antigen delivery to the MHC class I pathway. *Trends Immunol*. **25,** 92–97.

Mulvey, G.L., Marcato, P., Kitov, P.I., Sadowska, J., Bundle, D.R. and Armstrong, G.D. (2003). Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. *J. Infect. Dis*. **187,** 640–649.

Muniesa, M., Serra-Moreno, R. and Jofre, J. (2004). Free Shiga toxin bacteriophages isolated from sewage showed diversity although the stx genes appeared conserved. *Environ. Microbiol*. **6,** 716–725.

Nakagawa, I., Nakata, M., Kawabata, S. and Hamada, S. (1999). Regulated expression of Shiga toxin B gene induces apoptosis in mammalian fibroblastic cells. *Mol. Microbiol*. **33,** 1190–1199.

Nichols, B.J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol*. **4,** 374–378.

Noakes, K.L., Teisserenc, H.T., Lord, J.M., Dunbar, P.R., Cerundolo, V. and Roberts, L.M. (1999). Exploiting retrograde transport of Shiga-like toxin 1 for delivery of exogenous antigens into MHC class I presentation pathway. *FEBS Lett*. **453,** 95–99.

O'Loughlin, E.V. and Robins-Browne, R.M. (2001). Effect of Shiga toxin and Shiga-like toxins on eukaryotic cells. *Microbes. Infect*. **3,** 493–507.

Obrig, T.G., Seaner, R.M., Bentz, M., Lingwood, C.A., Boyd, B., Smith, A. and Narrow, W. (2003). Induction by sphingomyelinase of Shiga toxin receptor and Shiga toxin 2 sensitivity in human microvascular endothelial cells. *Infect. Immun*. **71,** 845–849.

Ohmura-Hoshino, M., Ho, S.T., Kurazono, H., Igarashi, K., Yamasaki, S. and Takeda, Y. (2003). Genetic and immunological analysis of a novel variant of Shiga toxin 1 from bovine *Escherichia coli* strains and development of bead-ELISA to detect the variant toxin. *Microbiol. Immunol*. **47,** 717–725.

Ohmura-Hoshino, M., Yamamoto, M., Yuki, Y., Takeda, Y. and Kiyono, H. (2004). Non-toxic Stx derivatives from *Escherichia coli* possess adjuvant activity for mucosal immunity. *Vaccine* **22,** 3751–3761.

Paton, A.W., Beutin, L. and Paton, J.C. (1995). Heterogeneity of the amino-acid sequences of *Escherichia coli* Shiga-like toxin type-I operons. *Gene* **129,** 87–92.

Paton, A.W., Morona, R. and Paton, J.C. (2000). A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans [see comments]. *Nature Med*. **6,** 265–270.

Paton, J.C. and Paton, A.W. (1998). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev*. **11,** 450–479.

Pierard, D., Muyldermans, G., Moriau, L., Stevens, D. and Lauwers, S. (1998). Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. *J. Clin. Microbiol*. **36,** 3317–3322.

Pijpers, A.H., van Setten, P.A., van Den Heuvel, L.P., Assmann, K.J., Dijkman, H.B., Pennings, A.H., Monnens, L.A. and van Hinsbergh, V.W. (2001). Verocytotoxin-induced apoptosis of human microvascular endothelial cells. *J. Am. Soc. Nephrol*. **12,** 767–778.

Pulz, M., Matussek, A., Monazahian, M., Tittel, A., Nikolic, E., Hartmann, M., Bellin, T., Buer, J. and Gunzer, F. (2003). Comparison of a Shiga toxin enzyme-linked immunosorbent assay and two types of PCR for detection of Shiga toxin-producing *Escherichia coli* in human stool specimens. *J. Clin. Microbiol*. **41,** 4671–4675.

Riederer, M.A., Soldati, T., Shapiro, J., Lin, J. and Pfeffer, S.R. (1994). Lysosome biogenesis requires Rab function and receptor recycling from endosomes to the trans-Golgi network. *J. Cell Biol*. **125,** 573–582.

Romisch, K. (1999). Surfing the Sec61 channel: bidirectional protein translocation across the ER membrane. *J. Cell Sci*. **112,** 4185–4191.

Saint-Pol, A., Yelamos, B., Amessou, M., Mills, I.G., Dugast, M., Tenza, D., Schu, P., Antony, C., McMahon, H.T., Lamaze, C. and Johannes, L. (2004). Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Dev. Cell* **6,** 525–538.

Salhia, B., Rutka, J.T., Lingwood, C., Nutikka, A. and Van Furth, W.R. (2002). The treatment of malignant meningioma with verotoxin. *Neoplasia*. **4,** 304–311.

Sandvig, K. (2001). Shiga toxins. *Toxicon* **39,** 1629–1635.

Sandvig, K., Garred, O., van Helvoort, A., van Meer, G. and van Deurs, B. (1996). Importance of glycolipid synthesis for butyric acid-induced sensitization to Shiga toxin and intracellular sorting of toxin in A431 cells. *Mol. Biol. Cell* **7,** 1391–1404.

Sandvig, K., Garred, Ø., Prydz, K., Kozlov, J.V., Hansen, S.H. and van Deurs, B. (1992). Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* **358,** 510–511.

Sandvig, K., Grimmer, S., Lauvrak, S.U., Torgersen, M.L., Skretting, G., van Deurs, B. and Iversen, T.-G. (2002). Pathways followed by ricin and Shiga toxin into cells. *Histochem. Cell Biol*. **117,** 131–141.

Sandvig, K., Olsnes, S., Brown, J.E., Petersen, O.W. and van Deurs, B. (1989). Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from Shigella dysenteriae 1. *J. Cell Biol*. **108,** 1331–1343.

Sandvig, K., Prydz, K., Ryd, M. and van Deurs, B. (1991). Endocytosis and intracellular transport of the glycolipid-binding ligand Shiga toxin in polarized MDCK cells. *J. Cell Biol*. **113,** 553–562.

Sandvig, K., Ryd, M., Garred, Ø., Schweda, E., Holm, P.K. and van Deurs, B. (1994). Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga B-fragment is regulated by butyric acid and cAMP. *J. Cell Biol*. **126,** 53–64.

Sandvig, K. and van Deurs, B. (2002). Transport of protein toxins into cells: Pathways used by ricin, cholera toxin, and Shiga toxin. *FEBS Lett*. **529,** 49–53.

Sandvig, K., Wälchli, S. and Lauvrak, S.U. (2004). Shiga toxins and their mechanism of cell entry. *Topics Curr. Genetics:* in press.

Sannerud, R., Saraste, J. and Goud, B. (2003). Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. *Curr. Opin. Cell Biol*. **15,** 438–445.

Schmidt, H. (2001). Shiga toxin-converting bacteriophages. *Res. Microbiol*. **152,** 687–695.

Schmidt, H., Montag, M., Bockemuhl, J., Heeseman, J. and Karch, H. (1993). Shiga-like toxin II-related cytotoxins in **Citrobacter freundii** strains from humans and beef samples. *Infect. Immun*. **61,** 534–543.

Schmidt, H., Scheef, J., Morabito, S., Caprioli, A., Wieler, L.H. and Karch, H. (2000). A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl. Environ. Microbiol*. **66,** 1205–1208.

Schmitt, C.K., McKee, M.L. and O'Brien, A.D. (1991). Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H-strain E32511. *Infect. Immun*. **59,** 1065–1073.

Schmitz, A., Herrgen, H., Winkeler, A. and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol*. **148,** 1203–1212.

Shimada, O., Ishikawa, H., Tosaka-Shimada, H. and Atsumi, S. (1999). Exocytotic secretion of toxins from macrophages infected with *Escherichia coli* O157. *Cell Struct. Funct*. **24,** 247–253.

Shimizu, T., Hamabata, T., Yoshiki, A., Hori, T., Ito, S., Takeda, Y. and Hayashi, H. (2003). An association of 27- and 40-kDa molecules with glycolipids that bind A-B bacterial enterotoxins to cultured cells. *Biochim. Biophys. Acta* **1612,** 186–194.

Shogomori, H. and Futerman, A.H. (2001). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. *J. Biol. Chem*. **276,** 9182–9188.

Shyng, S.L., Moulder, K.L., Lesko, A. and Harris, D.A. (1995). The N-terminal domain of a glycolipid-anchored prion protein is essential for its endocytosis via clathrin-coated pits. *J. Biol. Chem*. **270,** 14793–14800.

Siegler, R.L. (2003). Postdiarrheal Shiga toxin-mediated hemolytic uremic syndrome. *JAMA* **290,** 1379–1381.

Siegler, R.L., Obrig, T.G., Pysher, T.J., Tesh, V.L., Denkers, N.D. and Taylor, F.B. (2003). Response to Shiga toxin 1 and 2 in a baboon model of hemolytic uremic syndrome. *Pediatr. Nephrol*. **18,** 92–96.

Simpson, J.C., Roberts, L.M., Römisch, K., Davey, J., Wolf, D.H. and Lord, J.M. (1999). Ricin A chain utilizes the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett*. **459,** 80–84.

Sinha, G. (2003). Bacterial battalions join war against cancer. *Nat. Med*. **9,** 1229.

Smith, D.C., Gallimore, A., Jones, E., Roberts, B., Lord, J.M., Deeks, E., Cerundolo, V. and Roberts, L.M. (2002a). Exogenous peptides delivered by ricin require processing by signal peptidase for

transporter associated with antigen processing-independent MHC class I-restricted presentation. *J. Immunol*. **169,** 99–107.

Smith, D.C., Lord, J.M., Roberts, L.M., Tartour, E. and Johannes, L. (2002b). 1st class ticket to class I: protein toxins as pathfinders for antigen presentation. *Traffic* **3,** 697–704.

Smith, W.E., Kane, A.V., Campbell, S.T., Acheson, D.W., Cochran, B.H. and Thorpe, C.M. (2003). Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect. Immun*. **71,** 1497–1504.

Soltyk, A.M., MacKenzie, C.R., Wolski, V.M., Hirama, T., Kitov, P.I., Bundle, D.R. and Brunton, J.L. (2002). A mutational analysis of the globotriaosylceramide-binding sites of verotoxin VT1. *J. Biol. Chem*. **277,** 5351–5359.

Spilsberg, B., van Meer, G. and Sandvig, K. (2003). Role of lipids in the retrograde pathway of ricin intoxication. *Traffic* **4,** 544–552.

Sunyach, C., Jen, A., Deng, J., Fitzgerald, K.T., Frobert, Y., Grassi, J., McCaffrey, M.W. and Morris, R. (2003). The mechanism of internalization of glycosylphosphatidylinositol-anchored prion protein. *EMBO J.* **22,** 3591–3601.

Suzuki, M., Kondo, F., Ito, Y., Matsumoto, M., Hata, M., Oka, H., Takahashi, M. and Sakae, K. (2004). Identification of a Shiga-toxin type I variant containing an IS1203-like element, from Shiga-toxin producing *Escherichia coli* O157:H7. *FEMS Microbiol. Lett*. **234,** 63–67.

Tai, G., Lu, L., Wang, T.L., Tang, B.L., Goud, B., Johannes, L. and Hong, W. (2004). Participation of the syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the TGN. *Mol. Biol. Cell* **15,** 4011–4022.

Takenouchi, H., Kiyokawa, N., Taguchi, T., Matsui, J., Katagiri, Y.U., Okita, H., Okuda, K. and Fujimoto, J. (2004). Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells. *J. Cell Sci*. **117,** 3911–3922.

Tazzari, P.L., Ricci, F., Carnicelli, D., Caprioli, A., Tozzi, A.E., Rizzoni, G., Conte, R. and Brigotti, M. (2004). Flow cytometry detection of Shiga toxins in the blood from children with hemolytic uremic syndrome. *Cytometry* **61B,** 40–44.

te Loo, D.M., Monnens, L.A., van Der Velden, T.J., Vermeer, M.A., Preyers, F., Demacker, P.N., van Den Heuvel, L.P. and van Hinsbergh, V.W. (2000). Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. *Blood* **95,** 3396–3402.

Tetaud, C., Falguieres, T., Carlier, K., Lecluse, Y., Garibal, J., Coulaud, D., Busson, P., Steffensen, R., Clausen, H., Johannes, L. and Wiels, J. (2003). Two distinct Gb3/CD77 signaling pathways leading to apoptosis are triggered by anti-Gb3/CD77 mAb and verotoxin-1. *J. Biol. Chem*. **278,** 45200–45208.

Thorburn, A., Thorburn, J. and Frankel, A.E. (2004). Induction of apoptosis by tumor cell-targeted toxins. *Apoptosis*. **9,** 19–25.

Thorpe, C.M., Hurley, B.P., Lincicome, L.L., Jacewicz, M.S., Keusch, G.T. and Acheson, D.W. (1999). Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infect. Immun*. **67,** 5985–5993.

Thorpe, C.M., Smith, W.E., Hurley, B.P. and Acheson, D.W. (2001). Shiga toxins induce, superinduce, and stabilize a variety of C-X-C chemokine mRNAs in intestinal epithelial cells, resulting in increased chemokine expression. *Infect. Immun*. **69,** 6140–6147.

Torgersen, M.L., Skretting, G., van Deurs, B. and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci*. **114,** 3737–3747.

Tsai, B. and Rapoport, T.A. (2002). Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol*. **159,** 207–216.

Tsai, B., Rodighiero, C., Lencer, W.I. and Rapoport, T.A. (2001). Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* **104,** 937–948.

Uchida, T. (2003). STX-liposome conjugates as candidate vaccines. *Drugs Today (Barc.)* **39,** 673–693.

Urdahl, A.M., Beutin, L., Skjerve, E., Zimmermann, S. and Wasteson, Y. (2003). Animal host associated differences in Shiga toxin-producing *Escherichia coli* isolated from sheep and cattle on the same farm. *J. Appl. Microbiol*. **95,** 92–101.

Watanabe, M., Matsuoka, K., Kita, E., Igai, K., Higashi, N., Miyagawa, A., Watanabe, T., Yanoshita, R., Samejima, Y., Terunuma, D., Natori, Y. and Nishikawa, K. (2004). Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. *J. Infect. Dis*. **189,** 360–368.

Weinstein, D.L., Jackson, M.P., Samuel, J.E., Holmes, R.K. and O'Brien, A.D. (1988). Cloning and sequencing of a Shiga-like toxin type II variant from *Escherichia coli* strain responsible for edema disease of swine. *J. Bacteriol*. **170,** 4223–4230.

Wesche, J., Rapak, A. and Olsnes, S. (1999). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J. Biol. Chem*. **274,** 3443–3449.

White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. and Stelzer, H.K. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol*. **147,** 743–760.

Wiedlocha, A., Falnes, P.O., Madshus, I.H., Sandvig, K. and Olsnes, S. (1994). Dual mode of signal transduction by externally added acidic fibroblast growth factor. *Cell* **76,** 1039–1051.

Yamasaki, C., Natori, Y., Zeng, X.T., Ohmura, M., Yamasaki, S. and Takeda, Y. (1999). Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by non-toxic mutant Stx1, which lacks N-glycosidase activity. *FEBS Lett*. **442,** 231–234.

Ye, Y., Shibata, Y., Yun, C., Ron, D. and Rapoport, T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429,** 841–847.

Yoshida, T., Koide, N., Sugiyama, T., Mori, I. and Yokochi, T. (2002). A novel caspase dependent pathway is involved in apoptosis of human endothelial cells by Shiga toxins. *Microbiol. Immunol*. **46,** 697–700.

Zoja, C., Angioletti, S., Donadelli, R., Zanchi, C., Tomasoni, S., Binda, E., Imberti, B., te, L.M., Monnens, L., Remuzzi, G. and Morigi, M. (2002). Shiga toxin-2 triggers endothelial leukocyte adhesion and transmigration via NF-kappaB dependent up-regulation of IL-8 and MCP-1. *Kidney Int*. **62,** 846–856.

CHAPTER

# 18

# *Bacillus anthracis* toxins

*Stephen H. Leppla*

## INTRODUCTION

Anthrax was recognized for many centuries as a serious disease of animals and man, one that inflicted great losses in agricultural economies and caused significant disease in humans. Thus, anthrax was a major concern to the pioneers of microbiology, and its study by Pasteur, Koch, Mechnikoff, and others led to the establishment of many basic principles of infectious disease study. Regrettably, anthrax has regained a prominent role in infectious disease research following its use as a bioterrorism weapon in the United States in 2001 (Lane *et al*., 2001).

In domestic livestock and wild animals, symptoms of infection by *Bacillus anthracis* are rarely evident until the animal becomes lethargic several hours before death. Necropsy shows extensive edema in many tissues and concentrations of bacteria in blood that may exceed $10^8$ $mL^{-1}$ (Turnbull, 1990). Most human cases arise from contact with infected animals or spores present in animal products (wool, leather, bone meal), and begin as cutaneous infection. This is easily treated with antibiotics if correctly diagnosed. The less frequent but more dangerous gastrointestinal and respiratory forms of anthrax show a rapid progression and must be recognized early to be successfully treated (Inglesby *et al*., 2002).

The virulence of *B. anthracis* for animals and man depends on the production of two recognized virulence factors, the gamma-linked poly-D-glutamic acid capsule, and the three-component protein exotoxin (Smith *et al*., 1955; Keppie *et al*., 1963). The capsule appears to protect bacteria from phagocytosis, and therefore plays an essential role during establishment of an infection. Recent work suggests that release of capsular material from the bacteria may also contribute to pathogenesis (Makino *et al*., 2002). The protein exotoxin may also help to establish an infection by incapacitating phagocytes (Keppie *et al*., 1963; Wade *et al*., 1985; O'Brien *et al*., 1985), but its more obvious role is to cause the extensive tissue edema that appears to be a principal cause of death. It is generally accepted that the pathological effects causing death in infected animals result largely from the toxin (Smith and Stoner, 1967). A number of recent reviews have discussed the toxin and its role in pathogenesis (Turnbull, 1996; Dixon *et al*., 1999; Duesbery and Vande Woude, 1999; Little and Ivins, 1999; Leppla, 2000; Turnbull, 2002; Ascenzi *et al*., 2002; Mock and Mignot, 2003; Collier and Young, 2003; Mourez, 2004). A recent monograph includes six additional review chapters on anthrax (Koehler, 2002b).

Virulent strains of *B. anthracis* contain two large plasmids, pXO1 and pXO2. The genes coding for toxin are contained on pXO1 (Mikesell *et al*., 1983; Thorne, 1985), and the genes for capsule are on pXO2 (Uchida *et al*., 1985; Green *et al*., 1985). Virulence requires the presence of both plasmids. Thus, strains lacking plasmid pXO1 do not produce toxin and are essentially avirulent in most animals (Ivins *et al*., 1986; Uchida *et al*., 1986), but do retain some virulence for mice (Welkos *et al*., 1993). Strains lacking pXO2 are at least $10^5$-fold less virulent than wild-type (Ivins *et al*., 1986; Welkos and Friedlander, 1988). Although it is possible that these plasmids code for other materials that contribute to virulence, none that have a large role have been identified. Complete DNA sequences have been obtained for the plasmids and for the chromosomal DNAs of several strains (Read *et al*., 2003).

The anthrax toxins described in this chapter are studied both because they are the principal virulence determinants of *B. anthracis* and because they constitute a useful model for study of interactions of protein ligands with eucaryotic cells. A unique feature is that the three toxin proteins are individually non-toxic. These proteins are designated protective antigen (PA), lethal factor (LF), and edema factor (EF). Toxic activity is obtained only when the proteins are administered in pairwise combinations. The combination of PA with LF, which causes rapid death of certain animal species when injected intravenously, is designated lethal toxin (LT). The combination of PA with EF, which causes edema when injected intradermally, is designated edema toxin (ET).

Work in recent years, to be detailed in this chapter, shows that PA binds to receptors on eucaryotic cells (Bradley *et al*., 2001; Scobie *et al*., 2003) and mediates the internalization of LF and EF to the cytosol (Figure 18.1). EF is an adenylate cyclase; it converts ATP to unphysiologically high concentrations of cAMP that cause metabolic perturbations (Leppla, 1982; Leppla, 1984). LF is a metalloprotease that cleaves mitogen activated protein kinase kinases (MEKs, also MKKs) (Vitale *et al*., 1998; Duesbery *et al*., 1998). The anthrax toxins can be viewed as fitting the A/B model described by Gill (1978), where the A moiety is a catalytic polypeptide (i.e., enzyme), and the B moiety is the receptor binding region and also usually contains a subdomain that facilities membrane translocation. In the case of anthrax toxin, the PA protein serves as the B moiety, whereas LF and EF are alternate A moieties. Anthrax toxin is unusual in that the A and B moieties are separate gene products and proteins. Several other toxins are now known that have separate protein components. Most similar to anthrax toxins are a group of related clostridial toxins. In those toxins, the receptor recognition protein that resembles PA is activated by proteolysis and then binds and promotes the internalization of an actin ADP-ribosylating protein (Barth *et al*., 2004).

Some confusion may arise from use of the designation PA for a component of a toxin. Before discovery of the toxin, it was shown that culture supernatants of *B. anthracis* could immunize animals against infection (Gladstone, 1948). Only after the toxin was discovered did it become clear that the "protective antigen" in the culture supernates was a component of the toxin. PA remains the principal and essential immunogen in both killed and live anthrax vaccines (Hambleton *et al*., 1984; Ivins and Welkos, 1988). Therefore, study of anthrax toxin has direct application to development of improved vaccines, a subject not covered in depth here.

## THE GENETICS OF TOXIN AND VIRULENCE

### Plasmids pXO1 and pXO2

The possibility that the genes for the dominant *B. anthracis* virulence factors might be extrachromosomal was suggested by the work of Louis Pasteur and Max Sterne, who each isolated variants of *B. anthracis* having reduced virulence. After some initial difficulties, plasmids associated with toxin and capsule were discovered (Mikesell *et al*., 1983; Uchida *et al*., 1985; Green

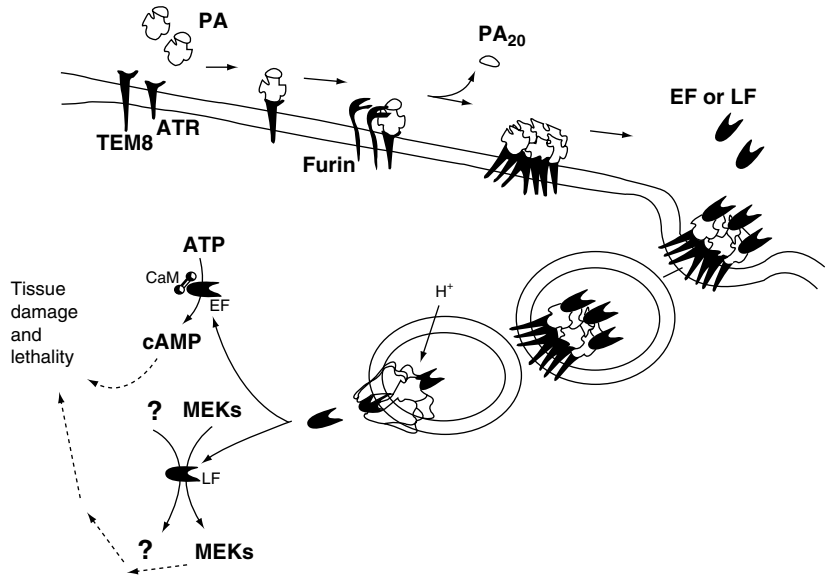



**FIGURE 18.1** Binding and internalization of anthrax toxin. Toxin components and cellular proteins that interact during LF and EF internalization are described in the text. CaM is calmodulin, and MEKs refers collectively to is mitogen activated protein kinase kinases.

*et al*., 1985). Special care is required in isolating these plasmids because their large size makes them easily sheared. Both these large plasmids have been sequenced. Plasmid pXO1 is 181.6 kb (Okinaka *et al*., 1999) and pXO2 is 96.2 kb (Pannucci *et al*., 2002). The replication origin of pXO2 is known to be similar to that of pAMβ1 (Tinsley *et al*., 2004), while that of pXO1 remains unknown.

The *B. anthracis* plasmids can each be selectively cured, pXO1 by repeated passage at 42°C, and pXO2 by growth in novobiocin. Comparison of plasmid-cured variants proved that pXO1 is needed for production of toxin and pXO2 for production of capsule (Thorne, 1985). Conjugal transfer of pXO1 to plasmid-cured strains of *B. anthracis* confirmed that all the genes necessary for toxin production are contained on the plasmid (Thorne, 1985; Heemskerk and Thorne, 1990). These results explain the properties and the efficacy of the anthrax vaccines developed by Louis Pasteur and Max Sterne. It is now well established that *B. anthracis* strains must produce PA in order to induce protective immunity (Ivins and Welkos, 1988). Elimination of the pXO1 plasmid yields an avirulent strain, but one that does not induce immunity. In retrospect, it is evident that Louis Pasteur's attenuation of the virulence of *B. anthracis* cultures by growth at 42°C was due to partial curing of plasmid pXO1. The cultures he used to successfully immunize sheep probably contained a small number of virulent ($pXO1^+$, $pXO2^+$) bacteria with a larger number of avirulent ($pXO1^-$, $pXO2^+$) bacteria, with their efficacy due to the former, which would induce antibodies to PA. While effective, these vaccines could cause infection if the fraction of virulent organisms was too high. Max Sterne's important contribution was to analyze carefully the rare, spontaneous non-capsulated variants appearing on agar plates, and to show that they were greatly reduced in virulence (Sterne, 1937). These variants, now known to have lost the pXO2 plasmid, are effective animal vaccines and do not revert to virulence. The ($pXO1^+$, $pXO2^-$) "Sterne" strain continues in use today as the preferred veterinary vaccine and as a convenient strain for laboratory studies because of its relative safety.

## Genes specifying toxin structure and expression

The recognition that anthrax toxin was encoded on pXO1 facilitated the cloning and sequencing of the genes encoding PA (*pagA*), LF (*lef*), and EF (*cya*) (Vodkin and Leppla, 1983; Robertson and Leppla, 1986; Tippetts and Robertson, 1988). Each of the genes has a G+C content of about 30% (Table 18.1), similar to that of the *B. anthracis* genomic DNA (35% G+C). Upstream of the ATG start codons in each of the genes is an appropriately located ribosome binding site, AAAGGAG for the PA and LF genes, and AAAGGAGGT for the EF gene. Each of the three proteins contains a typical bacillus signal peptide of 29–33 amino acids, with cleavage occurring after an Ala or Gly. Following the stop codon of the PA gene is an inverted repeat that may act as a transcriptional stop; no similar structures are present in the LF or EF regions.

Production of both capsule (Meynell and Meynell, 1964) and PA (Gladstone, 1948) by *B. anthracis* is dependent on addition of bicarbonate or $CO_2$. Early studies showed that stimulation of PA synthesis by bicarbonate requires the presence of a gene located on pXO1 (Bartkus and Leppla, 1989). This gene, *atxA*, was mapped by transposon mutagenesis and cloned and sequenced (Uchida *et al*., 1993; Koehler *et al*., 1994). The product of the *atxA* gene, a protein of 56 kDa, increases by at least 10-fold the transcription from a start site, P1, located at bp -58 relative to the start codon. Constitutive, low-level transcription initiates at another site, P2, at bp -26. Both these start sites are located in a potential 58-bp stem-loop structure (Welkos *et al*., 1988). Disruption of the *atxA* gene and complementation with an *atxA*-expressing plasmid proved that this regulator is needed for transcription of all three toxin genes, and it follows that *B. anthracis* strains lacking *atxA* are less virulent for mice (Uchida *et al*., 1993; Dai *et al*., 1995). A region of 111 bp upstream of the *pag* coding sequence is required for AtxA action, but no evidence is available suggesting that AtxA itself binds there (Dai *et al*., 1995) and no common sequences can be identified in the regions upstream of *pag*, *cya*, and *lef* to which

**TABLE 18.1** Properties of the anthrax toxin proteins

| | PA | LF | EF |
|---|---|---|---|
| %G+C in gene | 31% | 30% | 29% |
| AA residues in mature protein | 735 | 776 | 767 |
| AA residues in signal sequence | 29 | 33 | 33 |
| Sequence at signal peptide cleavage site[a] | IQA*E | VQG*A | VNA*M |
| Isoelectric point (calculated) | 5.6 | 6.1 | 6.8 |
| Gene accession numbers | M22589 | M29081 | M23179 |
| Structural accession numbers | 1ACC<br>1TZO[b]<br>1TZN[c]<br>1T6B[d] | 1J7N<br>1PWV[e] | 1K8T<br>1K93[f] |

[a]Asterisk shows site of signal peptide cleavage.
[b]PA heptamer
[c]PA heptamer bound to CMG2
[d]PA (monomer) bound to CMG2
[e]LF complexed to substrate peptide; additional LF complexes are not listed
[f]EF complexed with calmodulin; additional EF complexes are not listed

transcriptional regulators might bind. More recent studies with microarrays showed that AtxA is a key global regulator of many plasmid and chromosomal genes (Bourgogne *et al*., 2003; Mignot *et al*., 2004). The regulation of capsule biosynthesis by *atxA* requires the presence of either of two additional regulators, *acpA* and *acpB*, encoded on pXO2, and having limited sequence similarity to *atxA* (Drysdale *et al*., 2004).

## Toxin gene sequence homologies and variations

PA is similar to toxins produced by some pathogenic *Clostridia* and *Bacillus* species (Barth *et al*., 2004). All these homologues are binary toxins, like the anthrax toxins, but the second, catalytic component has ADP-ribosylation activity. The PA homologues are listed in Table 18.2, and the properties of the proteins are discussed in a later section. The most recent and novel addition to this list of proteins are two identified in an unusual *Bacillus cereus* strain, G9241, that contains a plasmid closely similar to pXO1 (Hoffmaster *et al*., 2004). This plasmid encodes proteins having high sequence identify to those in *B. anthracis* (99.7, 99, and 96% amino acid identity to PA, LF, and EF, respectively). A second large plasmid in the same strain, pBC218, contains additional PA and LF homologues resembling the *B. cereus* binary toxins mentioned above. It is not yet known whether these toxins are active. Comparison of these proteins may provide new insights into PA structure and function. This finding reminds us that *B. anthracis* and *B. cereus* are closely related and may represent the visible and more prominent parts of a genetic continuum.

Within the *B. anthracis* species, there is very little sequence variation between toxin genes of different isolates. Only three amino acid residues were found to vary among 26 sequenced PA genes (Price *et al*., 1999). It is of interest that the variable residues 536 and 571 are adjacent and on the surface of the protein, suggesting that these residues may be part of an epitope recognized by the immune system of animals and therefore under selective pressure to mutate to escape antibody neutralization.

The EF gene, *cya* (Escuyer *et al*., 1988;Robertson *et al*., 1988), has homology to several other adenylate cyclase genes of pathogenic bacteria (Ahuja *et al*., 2004), the best studied being that encoding the "invasive" adenylate cyclase of *Bordetella pertussis* (Glaser *et al*., 1988; Hanski and Coote, 1991). The homology to the *B. pertussis* cyclase occurs only in the regions known to comprise the catalytic domain. More recently, adenylate cyclases that may contribute to pathogenesis have been identified in *Pseudomonas aeruginosa* (Yahr *et al*., 1998) and in *Yersinia* species (Parkhill *et al*., 2001). The EF gene also has strong homology to the LF gene in the amino terminal region that is now recognized as involved in binding to PA. Beyond residue 250, LF has a very limited sequence homology to other metalloproteases.

## Role of the toxin in virulence

A number of tools are now available for performing genetic modifications to *B. anthracis* (Thorne, 1993; Koehler, 2002a). These include transduction and conjugation (Battisti *et al*., 1985; Heemskerk and Thorne, 1990) and transformation by electroporation (Bartkus and Leppla, 1989). The elegant methods developed for use of Tn*917* in *Bacillus subtilis* have been adapted to *B. anthracis* (Heemskerk and Thorne, 1990). Methods for gene disruptions are also available (Dai *et al*., 1995). A conjugational transfer system was used to transfer a mutated PA gene into the Sterne strain, replacing the resident PA gene (Cataldi *et al*., 1990). The latter

**TABLE 18.2** Protein neighbors of PA

| Protein | Accession numbers | Reference |
|---|---|---|
| *Clostridium perfringens* iota toxin component Ib | X73562 (gene)<br>CAA51959 (protein) | (Perelle *et al*., 1995) |
| *Clostridium spiroforme* toxin component Sb | X97969 (gene)<br>CAA66611 (protein) | (Popoff and Boquet, 1988) |
| *Clostridium difficile* binary toxin component CDTb | L76081 (gene)<br>AAB67304 (protein) | (Perelle *et al*., 1997) |
| *Clostridium botulinum* C2 toxin component II | D88982 (gene)<br>BAA32537 (protein) | (Kimura *et al*., 1998) |
| *Bacillus thuringiensis* vegetative insecticidal protein (vip1Ac) | AY245547 (gene)<br>AAO86514 (protein) | (Shi *et al*., 2004) |
| *Bacillus cereus* protective antigen (from strain G9241, plasmid pBCX01) | (not available) | (Hoffmaster *et al*., 2004) |
| *Bacillus cereus* protective antigen (from strain G9241, plasmid pBC218) | AAEK01000004 (pBC218)<br>EAL15944 (protein) | (Hoffmaster *et al*., 2004) |

method was employed to produce strains expressing every combination of the three components (Table 18.3). The only strain that retained some virulence was RP9, which makes PA and LF (Pezard *et al*., 1991; Pezard *et al*., 1993). This suggests that LF is the more important virulence factor, whereas EF provides a smaller contribution. However, this analysis needs to be repeated in a strain having pXO2 in addition to pXO1 because of the interaction of regulatory genes on the two plasmids (Bourgogne *et al*., 2003).

## THE PROTEINS

### Production of toxin from *B. anthracis* and *B. subtilis*

Study of the anthrax toxin proteins has been facilitated by the relative ease with which they can be prepared in milligram amounts. Extensive work in the period 1940–1965 led to development of synthetic media that support good production of PA for use in vaccines (Puziss *et al*., 1963; Haines *et al*., 1965). Key ingredients of synthetic media are bicarbonate (Gladstone, 1946) to activate toxin gene transcription (Bartkus and Leppla, 1989) and a buffering system that maintains the pH above 7.0 (Strange and Thorne, 1958). Later work led to development of a completely synthetic medium, designated R (Ristroph and Ivins, 1983), which was subsequently modified further (Leppla, 1988; Leppla, 1991a). Growth of 50-L fermentor batches typically yielded 500 mg PA, 100 mg LF, and 40 mg EF.

For production of individual toxin proteins and mutated variants of them, the PA gene and upstream sequences were cloned into the staphylococcal vector pUB110, which is a high copy number plasmid in bacilli. The resulting vectors, pPA101 and pPA102, expressed PA at 20–40 mg/L from *Bacillus subtilis*, but the PA was rapidly destroyed by extracellular proteases (Ivins and Welkos, 1986). Expression from pPA101 in *B. subtilis* WB600, a strain in which six extracellular proteases are inactivated, improved yields of intact PA (Miller *et al*., 1998), but degradation of PA in the WB600 cultures remained a problem compared to the situation in *B. anthracis* culture supernatants, which are quite low in secreted proteases. In the most extensive studies with the pPA102 plasmid, use of a sporulation deficient *B. anthracis* strain grown in a rich medium under optimized fermentor conditions yielded PA at 20–30 mg/L (Farchaus *et al*., 1998).

A further improvement for production of PA and LF in *B. anthracis* came with creation of the shuttle vector pYS5, constructed from pPA102 and a pBR322-derived plasmid (Singh *et al*., 1989). This vector lacks the *atxA* regulator gene, causing transcription from the normally used PA promoter to be low, and yields in RM medium are minimal. However, in a rich medium (FA) under optimal conditions, PA is produced at 50–100 mg/L. In this case, transcription may originate from other promoters. The pYS5 vector has been used successfully in the author's lab and by others to produce PA and many PA mutants. Efficient production of LF has been achieved in a similar expression vector containing the PA promoter and signal peptide (Park and Leppla, 2000). A comparable vector for EF production has proved less effective. In general, these findings emphasize the advantages of producing a protein in its original host, where there has been natural selection to adapt the toxin to the host's secretion apparatus and to resist the host's proteases.

### Production of toxin components and fusion proteins in *E. coli*

For production of native and mutated anthrax toxin proteins, many laboratories have used *E. coli* expression systems. T7-based vectors that include signal peptides were found to successfully secrete PA and PA mutant proteins to the *E. coli* periplasm with

**TABLE 18.3** Virulence properties of modified *B. anthracis* strains

| Strain name | Parental strain | Plasmid content | Proteins produced | $LD_{50}$ for mice |
|---|---|---|---|---|
| 7702 | - | pXO1 | PA, LF, EF | $10^6$ |
| 7700 | 7702 | -(cured) | | $>10^9$ |
| RP8 | 7702 | pXO1 pagΔ322 | LF, EF | $>10^9$ |
| RP9 | 7702 | pXO1 cyaΔ303 | PA, LF | $10^7$ |
| RP10 | 7702 | pXO1 lefΔ238 | PA, EF | $>10^9$ |
| RP4 | RP9 | pXO1 cyaΔ303 pagΔ652 | LF | $>10^9$ |
| RP31 | RP10 | pXO1 lefΔ238 pagΔ652 | EF | $>10^9$ |
| RP42 | RP10 | pXO1 lefΔ238 cyaΔ303 | PA | $>10^9$ |

Toxin genes were inactivated by insertion of erythromycin or kanamycin resistance genes (Cataldi *et al.*, 1990; Pezard *et al.*, 1991; Pezard *et al.*, 1993). Swiss mice were injected subcutaneously with spore suspensions and lethality monitored.

yields after purification of 0.5 mg/L (Sharma *et al*., 1996; Benson *et al*., 1998). A T5-based vector was used to produce LF with an N-terminal, 6-His sequence, which enabled convenient affinity purification at yields of 1.5 mg/L (Gupta *et al*., 1998). With PA and LF, use of synthetic genes having codon usage suitable for *E. coli* has improved yields to high levels (Laird *et al*., 2004). Fusion proteins in which LF residues 1–254 (LFn) are attached to other polypeptides have been made in several standard *E. coli* expression systems (Arora and Leppla, 1993; Ballard *et al*., 1996). Initial EF expression work produced a protein lacking the N-terminal 261 aa that was expressed at 2% of total protein in *E. coli*, purified to homogeneity, and used in characterizations of the catalytic properties (Labruyere *et al*., 1990). More recently, full-size EF has been produced from *E. coli* in good yields (Kumar *et al*., 2001; Soelaiman *et al*., 2003; Cooksey *et al*., 2004).

## Toxin purification

The toxin proteins collectively, or the recombinant PA and LF proteins individually, constitute more than 50% of the protein present in *B. anthracis* culture supernatants grown in the R or FA media noted above. This makes purification of the PA and LF proteins relatively easy once the proteins have been protected from proteases and concentrated. Recovery from culture supernatants has been done by hydrophobic "salting out" onto agarose resins (Leppla, 1991a), but other methods can also be used. Effective purification steps include chromatography on anion exchange resins or hydroxyapatite. Detailed protocols for purification from *B. anthracis* are available (Quinn *et al*., 1988; Leppla, 1988; Leppla, 1991a; Farchaus *et al*., 1998; Park and Leppla, 2000; Ramirez *et al*., 2002). Methods used for purification of the toxin proteins from *E. coli* have been more diverse. However, because the proteins lack disulfide bonds, they can be refolded from inclusion bodies in good yields. *E. coli* expression systems have the disadvantage that the products need to be analyzed for endotoxin contamination.

## Structural features common to the three toxin components

All three of the anthrax toxin proteins are similar in size and charge (Table 18.1). Especially notable is that all three proteins lack cysteine. This has proved advantageous for structure function analyses, because any cysteine added by mutagenesis for subsequent chemical modification is unique (Nassi *et al*., 2002; Mourez *et al*., 2003). It was noted some years ago that extracellular bacterial proteins generally have a low cysteine content (Pollack and Richmond, 1962), and this generalization appears to hold for a number of other secreted bacterial toxins. Perhaps most striking is the absence of cysteines in the *B. pertussis* adenylate cyclase, a protein of 1706 residues (Glaser *et al*., 1988).

## Overview of toxin binding and internalization by cells

Data on toxin structure combined with studies on interaction with cells to be discussed below has led to a model of toxin uptake depicted in Figure 18.1 (Petosa *et al*., 1997; Moayeri and Leppla, 2004). PA binds to cell surface receptors and is cleaved by cell surface proteases, principally furin, with release of the 20-kDa N-terminal fragment. PA63 then oligomerizes and also binds LF or EF. The complex is internalized by endocytosis, and acidification of the vesicle causes insertion of the PA63 heptamer into the endosomal membrane to produce a channel through which LF or EF translocate to the cytosol. Details of the individual steps are discussed in later sections.

## PA structure and function

### *PA crystal structure*

The structure of PA was solved by X-ray diffraction (Petosa *et al*., 1997). PA is a long, flat protein that is rich in β-sheet structure (Figure 18.2). Four domains are distinguished that correspond to functional regions previously defined by analysis of large fragments produced by trypsin and chymotrypsin. Domain 1 (aa 1-258) contains two tightly bound calcium ions and a large flexible loop (aa 162–175) that includes the sequence $_{164}$RKKR$_{167}$ that is cleaved during proteolytic activation. Domain 2 (aa 259–487) contains several very long β strands and forms the core of the membrane-inserted channel. It also has a large flexible loop (aa 303–319) implicated in oligomer formation and membrane insertion. Domain 3 (aa 488–595) has no clearly defined function. Domain 4 is loosely associated with the other three domains and is involved in receptor binding.

### *PA proteolytic activation, nicking, oligomerization, and binding of LF and EF*

The two flexible loops mentioned above contain sites uniquely sensitive to proteolytic cleavage, as was recognized even before the aa sequence was known. The sequence $_{164}$RKKR$_{167}$ is extremely sensitive to cleavage by trypsin, clostripain, and other proteases that recognize basic residues. PA in solution at 1 mg mL$^{-1}$ is completely cleaved in 30 minutes when treated with 0.1 μg mL$^{-1}$ of trypsin (Leppla *et al*., 1988; Singh *et al*., 1989).

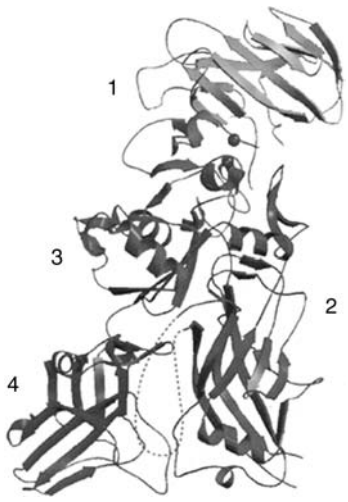



**FIGURE 18.2** Structures of anthrax toxin protective antigen (PA). PA domains 1–4 are indicated.

The fragments of 20 and 63 kilodaltons (designated PA20 and PA63, respectively) do not easily dissociate. However, at pH 7.5, concentrated solutions of the nicked PA incubated for several hours form a precipitate, which can be shown by SDS gel electrophoresis to contain PA63. Chromatography of trypsin-treated PA on the MonoQ anion exchange resin (Pharmacia) at pH 9.0 yields an early peak of PA20 and a later peak containing PA63. PA63 purified on MonoQ resin remains soluble indefinitely if kept at pH 9.0. Analysis by nondenaturing gel electrophoresis and gel filtration chromatography showed this material to be a large oligomer of the 63-kDa peptide (Leppla *et al*., 1988; Singh *et al*., 1994). This oligomer is extremely stable. By transmission electron microscopy after negative staining, the oligomer was shown to be a heptamer (Milne *et al*., 1994). This species was crystallized and its structure determined by X-ray diffraction, which confirmed the heptameric nature of the oligomer (Petosa *et al*., 1997). More recently, a higher resolution structure of the heptamer bound to receptor was determined (Lacy *et al*., 2004a). This showed that the loop including residues 303–309 stabilizes the heptamer by closely associating with neighboring PA63 monomers in the heptamer.

The functional importance of the trypsin-sensitive site at residues 164–167 became evident when it was noted that PA incubated with cells becomes nicked at this site (Leppla *et al*., 1988). Only PA63 remains bound to cells; PA20 can be detected in the supernate if this is concentrated before analysis. PA cleavage on cells occurs at 4°C, a condition in which PA is retained on the cell surface. A PA mutant in which the $_{164}$RKKR$_{167}$ sequence is deleted is not cleaved on the surface of cells and is non-toxic (Singh *et al*., 1989). The implication that PA63 is the active species needed for delivery of LF or EF was proven directly by showing that purified, heptameric PA63 is toxic to macrophages when combined with LF. Finally, PA incubated with cells at 37°C and allowed to internalize forms oligomers that are stable to heating in SDS (Milne *et al*., 1994; Liu and Leppla, 2002). This occurs because acidification of endosomes causes the oligomer to insert into membranes to form a second, stable type of heptamer (to be discussed later).

A detailed analysis of PA residues 164–167 showed that cleavage by cellular proteases requires the minimum sequence RxxR (Klimpel *et al*., 1992). This result, combined with inhibitor studies, proved that the cellular protease that most rapidly activates PA is furin. This finding was consistent with evidence that a number of other bacterial toxins require proteolytic activation by furin (Gordon *et al*., 1995; Gordon *et al*., 1997). The strict requirement that PA be proteolytically cleaved on the cell surface has been exploited in the design of toxins dependent on other proteases, as will be discussed later.

The other site that is uniquely sensitive to protease is the large loop in domain 2, aa 303–319. Cleavage in this region occurs in *B. anthracis* culture supernates, probably due to the action of a metalloprotease of the thermolysin type. Cleavage at the same site is obtained with 1 μg mL$^{-1}$ of chymotrypsin or thermolysin. This cleavage occurs at the pair of Phe residues, aa 313–314 in the sequence SFFDI (Novak *et al*., 1992). Deletion of the pair of Phe completely inactivates PA (Singh *et al*., 1994). The deleted mutant PA binds to cells, becomes nicked, and internalizes LF to endosomes, but fails to tranlocate LF to the cytosol (Novak *et al*., 1992; Singh *et al*., 1994). PA proteins mutated at this site have served as valuable controls when determining whether action of PA variants requires access to the cytosol. Receptor binding studies using radiolabeled PA that was nicked at residues 313–314 showed that only the C-terminal 47-kDa fragment was retained on cells. This result showed that the cell recognition domain is entirely contained in the 47-kDa fragment, residues 315–735.

The PA63 heptamer binds tightly to LF, as can be demonstrated by several methods, including sedimentation equilibrium and gel electrophoresis. On nondenaturing 5% polyacrylamide gels run at pH 8.5, PA63 moves as a sharp band, much slower than PA. In mixtures of PA63 and LF, several very closely spaced bands migrating even more slowly than PA63 are seen. These contain oligomeric PA63 with increasing

numbers of bound LF molecules (Singh *et al*., 1999). Although the author's initial studies were interpreted as showing that a PA63 heptamer can bind seven LF molecules, strong evidence against this came from mutagenesis studies showing both that the LF/EF binding site spans two adjacent PA63 monomers (Mogridge *et al*., 2002b) and that the large footprint of LF/EF on the heptamer precludes simultaneous use of two adjacent binding sites (Cunningham *et al*., 2002; Mogridge *et al*., 2002a). It follows that a maximum of three LF/EF molecules can bind to the heptamer. However, in recent cryo-electron microscopy analyses, only a single LF was observed bound to the PA63 heptamer (Ren *et al*., 2004), indicating that the stoichiometry question deserves continuing study.

### *Functional sites of PA defined by mutagenesis*

*Domain 1*

Removal of residues 1–167 (domain 1a) by proteolytic activation and formation of the heptameric PA63 leaves a surface (domain 1b) to which LF and EF bind. Two groups have used mutagenesis to identify PA residues involved in LF binding. Nine residues on the surface that is thought to interact with LF were altered, and four of them (aa 202, 203, 205, and 207) were found to be essential (Chauhan and Bhatnagar, 2002). A more extensive mutagenesis study agreed that aa 205 and 207 are required for LF binding, and implicated five additional residues (Cunningham *et al*., 2002). More importantly, by introducing certain of the mutations into separate monomers that could only form dimers, the residues involved in the adjacent monomer's subsites of the LF binding site were identified.

*Domain 2*

The crystal structure shows that residues 259–487 form the central core of the heptameric PA channel. Recognition that the large loop including aa 303–319 had alternating hydrophilic and hydrophobic residues led to the proposal that its insertion into the membrane would produce a β barrel (Figure 18.3) like that seen in the structure of staphylococcal alpha-hemolysin (Petosa *et al*., 1997). Extensive mutagenesis of residues 302–325 confirmed that the hydrophobic side chains interact with lipid while the hydrophilic residues are accessible to solvent, as expected if the structure followed the example of the alpha-hemolysin structure (Benson *et al*., 1998). Subsequent studies extended this analysis and suggested that the β barrel involved the entire region from aa 275 to 352 (Nassi *et al*., 2002), implying that a major rearrangement of the heptamer takes place upon membrane insertion, and that the β barrel extends beyond the bilayer.

The requirement for oligomerization implies that dominant negative mutant proteins can exist, ones in which mutations within a single monomer could inactivate an entire heptamer. A systematic mutagenesis study of the entire PA63 region was designed to identify candidate dominant negative mutants (Mourez *et al*., 2003). At least 33 residues in the entire PA63 sequence were found to be essential for toxicity. A more targeted search for dominant negative mutants identified several having that behavior (Sellman *et al*., 2001; Singh *et al*., 2001). By definition, such mutants are able to form the heptameric prepore, but this fails to convert to a functional protein-conducting channel. In theory, one could also expect that mutation of key residues lining the lumen of the channel may also yield dominant negative proteins, but none have yet been reported.

Systematic mutagenesis of domain 2 also identified several residues within the extended β barrel region that were required for toxicity (Mourez *et al*., 2003). Of particular interest are residues 337, 342, and 346, which were later shown to participate in binding of the prepore heptamer to the cellular receptor (Santelli *et al*., 2004; Lacy *et al*., 2004a), as will be discussed later.

*Domain 3*

Systematic mutagenesis studies (Mogridge *et al*., 2001; Mourez *et al*., 2003) identified very few residues in domain 3 (aa 488–595) that are essential for activity. An E515C mutant was inactive but an E515A mutant retained activity. The loops containing aa 483–486 and 510–518 interact with the adjacent monomer in the

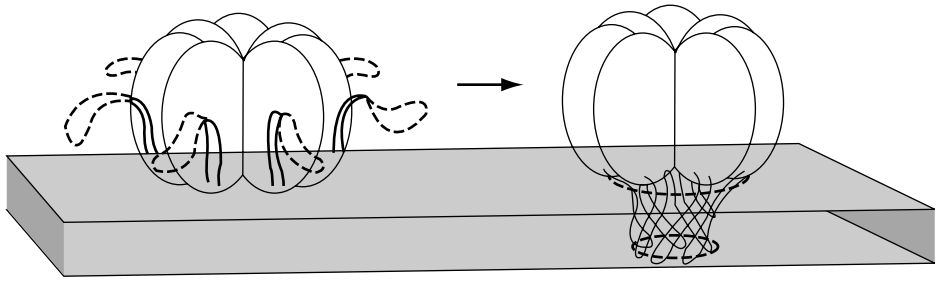


**FIGURE 18.3** Model for insertion of amphipathic hairpins to produce β barrel channel. The heptameric PA63 is shown with the flexible loop containing residues 300–325, shown as a dotted line.

heptamer prepore structure (Lacy *et al*., 2004a), and substitutions in these loops had modest effects on function. However, evidence to date suggests that the principal role of domain 3 is to stabilize the heptamer. It can be noted that the only residues (aa 536 and 571) that vary among the natural *B. anthracis* PA isolates (see above) are located in domain 3. If these residues are a target of neutralizing antibodies, as suggested above, then those antibodies might neutralize by blocking heptamer formation.

*Domain 4*

The C-terminal domain 4 of PA was initially implicated in receptor binding by analysis of mutants truncated to varying extents (Singh *et al*., 1991). Later studies suggested that the effect of these truncations was indirect, perhaps by causing changes to the folding of domain 4. Codon-based mutagenesis was used to identify important residues in the two solvent-exposed loops of domain 4 that were predicted to interact with the cellular receptor (Varughese *et al*., 1999; Brossier *et al*., 1999). Substitution of N657 or N682 had the largest effects in decreasing PA binding to receptor. Further mutagenesis of the small loop containing residues 679–693 showed that this region contains four residues for which single alanine substitutions decreased toxicity more than 10-fold (Rosovitz *et al*., 2003). The D683 residue was shown to be critical, which was later explained by its interaction with the divalent metal ion in the receptor, as discussed later. The systematic mutagenesis study referenced above (Mourez *et al*., 2003) identified residues 656, 657, 665, 682, 683, and 687 as important for PA activity.

### *Functional sites of PA defined by monoclonal antibodies*

Mouse monoclonal antibodies to PA were assigned by competition to at least 23 different epitopes (Little *et al*., 1988). However, very few of these antibodies neutralized the toxin. Three antibodies, including 14B7 and 3B6, react with a region that overlaps with the receptor-binding domain. Thus, 14B7 blocks binding of radiolabeled PA to cells and neutralizes toxicity when mixed with PA prior to addition to cells. Detailed mapping with alanine mutants in the domain 4 small loop showed that the sets of residues involved in binding to receptor and to 14B7 overlapped, but were not identical (Rosovitz *et al*., 2003). A number of companies have recently developed human antibodies to PA as therapeutic agents, and several of these appear to react with domain 4 (Sawada-Hirai *et al*., 2004).

Monoclonal antibodies may also act by blocking proteolytic activation of PA or oligomerization of PA63. An example of the former is provided by a mouse antibody to PA, which appears to prevent furin cleavage (Brossier *et al*., 2004). Well-characterized examples of antibodies that block heptamerization have not yet been reported.

The model for interaction of PA63 with LF predicts that neutralizing monoclonal antibodies could also act by blocking LF and EF binding to PA63. One antibody obtained by immunization with PA63 does have this property. Antibody 1G3 is unique because it reacts with PA63 but not with PA, it blocks LF binding to PA63, and it neutralizes toxin even when added at amounts far below those of PA (Little *et al*., 1996). This high potency occurs because 1G3 reacts only with the PA63 generated by activation of receptor-bound PA and not with the intact PA that is not bound to cells. The 1G3 antibody is also the only one of the mouse monoclonal antibodies tested that was able to delay the time to death of guinea pigs challenged with virulent *B. anthracis* (Little *et al*., 1997). A human antibody reacting to this same LF binding site was selected by panning of a phage-displayed antibody library on PA63, while adding soluble PA to block binding to other epitopes (Wild *et al*., 2003). This antibody neutralized toxin at less than molar ratios, like the mouse 1G3 antibody, and it protected rats from toxin challenge.

Surprisingly, none of the more than 35 monoclonal antibodies obtained by immunization with PA was reactive with PA20. Cleavage of PA on the surface of cells may release PA20 as a peptide that is too small to efficiently induce antibody. Monoclonal antibodies to PA20 were later obtained by immunizing mice with purified PA20 (unpublished work of Little and Leppla).

### *Conservation of functional sites within neighbors of PA*

The proteins having homology to PA are listed in Table 18.2. The high degree of similarity of these proteins argues that all are derived from a common ancestor. It is expected that these proteins all share the same structural design as PA. All six proteins have perfect conservation of the five Asp and Glu residues in PA whose side chain carboxyl groups chelate the two calcium ions (Figure 18.4). While several of these proteins are known to require proteolytic activation, none have a fully furin-susceptible cleavage site. The *C. spiroforme* toxin has a minimum furin site, RSAR, and several of the other proteins have basic residues in the putative cleavage region. Perhaps the four clostridial toxins, because they are produced by anaerobic pathogens that grow in restricted spaces, have evolved to depend on activation by proteases of the producing bacteria.

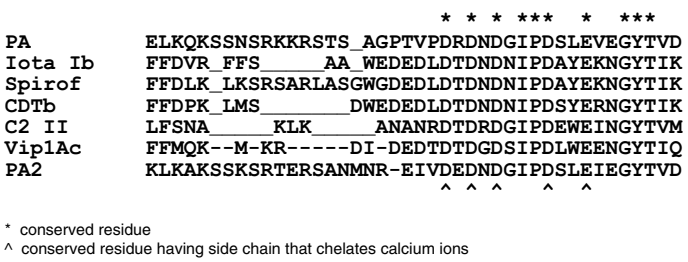



**FIGURE 18.4** Sequences of PA family members in the region containing the protease activation and calcium binding sites. Protein designations correspond to those in Table 18.2. Vip1Ac is vegetative insecticidal protein 1 of *B. thuringiensis*. PA2 is the protein encoded by plasmid pBC218 of *B. cereus* G9241.

Sequence similarity of the proteins decreases greatly in the regions corresponding to PA domain 4, suggesting that the toxins have diverged from a common ancestor so as to acquire specificity for cellular receptors in different target organisms. Little is known about the cellular receptors for these toxins. The C2 toxin receptor appears to require asparagine-linked carbohydrates, but no protein component has been identified. Progress in understanding these toxins would be greatly aided by identifying their cellular receptors.

## EF structure and function

### *EF structure*

EF was the last of the three toxin components to have its structure solved, and initially only the structure of the catalytic domain was reported (Drum *et al.*, 2002). Comparison of the LF and EF sequences showed that the N-terminal regions of approximately 250 residues have substantial homology, consistent with their common ability to bind to PA63. The EF region following residue 250 has homology to the *B. pertussis* cyclase and is an active enzyme when expressed as a recombinant protein (Labruyere *et al.*, 1991). Adenylate cyclase activity absolutely requires the presence of calmodulin (Leppla, 1984), and the basis for this was evident in the intimate association of the two proteins and the large changes induced in the EF structure upon calmodulin binding (Drum *et al.*, 2002). In particular, the C-terminal largely helical domain (aa 660–800) undergoes major rearrangements when associated with calmodulin. Three other regions, termed switches A, B, and C, are rearranged in the EF-calmodulin complex so as to form the catalytic site. The structure of the calmodulin in the complex is unusual in that the C-terminal domain has two bound calcium ions, as it does in its interactions with other proteins, whereas the N-terminal domain, which has a similar overall structure, is altered by its strong association with EF so that it does not bind calcium. Extensive mutagenesis and NMR studies suggested a model in which the N-terminal calmodulin domain associates weakly with EF and brings the C-terminal domain close to EF. Binding of calcium ions to the C-terminal domain gives it the ability to associate strongly with the other EF domains and stabilize the rearranged, catalytically active EF species (Shen *et al.*, 2002; Ulmer *et al.*, 2003). These studies also identified the active site residues involved in catalysis and characterized the complex role of calcium in EF activation.

Information about the structure of EF was also deduced from a set of monoclonal antibodies (Little *et al.*, 1994). Cleavage in formic acid produced the three fragments of 18, 53, and 17 kDa expected from cleavage at Asp-Pro bonds, and these were used to map the antibodies. The two antibodies that blocked binding of EF to PA on cells reacted with the N-terminal 18-kDa fragment, consistent with its role in binding to PA. Several other antibodies inhibited adenylate cyclase activity.

### *EF catalytic activity*

After initial reports on the catalytic properties of EF (Leppla, 1982; Leppla, 1984; Labruyere *et al.*, 1991), there was a period of inactivity, only recently ended. This is surprising because the enzyme is highly active and therefore has considerable potential as a pharmacological tool for transiently increasing cAMP concentrations in eucaryotic cells. EF has high catalytic activity, with a $V_{max}$ = 1.2 mmol cAMP/min/mg protein (Leppla, 1984), corresponding to a turnover number of 1000–2000 molecules per second. The Km for ATP in the presence of $Mg^{2+}$ is 0.16 mM. Similar values have been reported in more recent studies (Shen *et al.*, 2002). The enzyme activity is very sensitive to $Ca^{2+}$, showing optimum activity at 1–100 μM (depending on assay conditions) and inhibition at higher concentrations. $Mn^{2+}$ can substitute for $Ca^{2+}$ in activation of calmodulin and does not cause inhibition, so its use is convenient in routine assays to make the assay insensitive to variations in free $Ca^{2+}$.

The enzyme activity of EF has an absolute requirement for calmodulin. In the presence of 50 μM $Ca^{2+}$, the concentration of calmodulin giving half-maximal activity is 2.0 nM. When the $Ca^{2+}$ is chelated by excess EGTA, calmodulin still can activate EF, but 5 μM is needed to get equivalent activity. Thus, calmodulin can

activate EF even when it contains no bound $Ca^{2+}$. The *B. anthracis* cyclase differs from the *B. pertussis* enzyme in that fragments of the protein have no activity in the absence of calmodulin. This is consistent with the observation that *E. coli* strains expressing EF but not calmodulin produced no detectable cAMP and do not have any adenylate cyclase activity (Mock *et al.*, 1988), in spite of the probable presence of breakdown fragments of the EF protein.

The availability of the crystal structure of EF has enabled a detailed analysis of the catalytic process (Guo *et al.*, 2004; Shen *et al.*, 2004). A structure of EF bound to cAMP and pyrophosphate was used to propose that catalysis involves a key histidine residue and two divalent ions. It was also shown that the reverse synthetic reaction to produce ATP can proceed at the same rate as the forward reaction, suggesting that EF might have value for synthesis of ATP analogs from modified cAMP compounds. In the structure obtained for the complete EF protein, the structure of the N-terminal PA-binding domain was shown to be very similar to the structure of the corresponding LF domain, as was expected from their similarity in sequence and function (Shen *et al.*, 2004).

## LF structure and function

### *LF structure*

Although biochemical analyses had provided an overview of the functional domains, solving the crystal structure of LF provided the information needed to greatly accelerate study of its function (Pannifer *et al.*, 2001). The structure showed that the N-terminal domain I, aa 1–262, is an entirely separate domain having no interactions with the rest of the protein. This is consistent with its role in binding to the PA heptamer. Mutagenesis of surface residues conserved between LF and EF identified a local surface region formed by two sequences (aa 182–188 and aa 223–236) in which seven different single alanine substitutions greatly reduced binding to PA (Lacy *et al.*, 2002). The same mutations introduced into a corresponding EF construct produced very similar effects on binding. Other studies have identified additional residues that are important for LF activity, but in these cases, it was possible that the mutations altered folding of the domain (Quinn *et al.*, 1991; Gupta *et al.*, 2001; Kumar *et al.*, 2001).

Domain 2, containing residues 263–297 and 385–550, has limited sequence similarity but high structural homology to ADP-ribosylating toxins such as C2 and Vip, the same toxins in which the second, cell-binding component resembles PA (Table 18.2). This domain does not retain key residues needed for NAD binding and catalysis. This unexpected finding strongly suggests that LF arose from the homologous clostridial or bacillus toxins by a recombinational event that captured the gene encoding a metalloprotease. Inserted into the middle of domain 2 is domain 3 (aa 298–384), which consists of four imperfect repeats of a 19-amino acid sequence that forms a helix-turn unit. This sequence closely matches a sequence within domain 2, indicating that it arose by repeated duplication of that region. Comparison of the sequences of the repeats suggests that repeat 1 (in domain 2) was duplicated to make repeat 2, repeat 2 was duplicated to make repeat 3, and then the pair 2+3 was duplicated to make 4+5. This suggests that there was continuing selective pressure to increase the size of this domain. The resulting helical bundle lies across domain 4, thereby greatly restricting steric access to the protease active site and contributing to the extremely limited substrate specificity.

Domain 4 contains the catalytic domain. The recognition that the LF sequence contains a site characteristic of zinc metalloproteases (Klimpel *et al.*, 1994) started a process that eventually led to the identification of its catalytic activity. The sequence of the entire domain is only distantly related to other zinc-dependent proteases, and the similarity was too low to be recognized by the standard homology searching programs. It is interesting in retrospect that LF has greatest sequence similarity at the active site with the *B. cereus* NprB protease, both having a HEFGHAV sequence that includes the zinc-binding HExxH motif. The limited sequence similarity suggested that LF is a zinc metalloprotease. This was supported by showing that substitution of Ala for H686, E687, or H690 in the sequence $_{686}$-HEFGH-$_{690}$ destroyed the toxicity of LF for macrophages and decreased its zinc binding ability (Klimpel *et al.*, 1994). Attempts to find substrates cleaved by LF extended over several years until the independent efforts of two labs showed MEKs to be cleaved (Vitale *et al.*, 1998; Duesbery *et al.*, 1998), as discussed below.

It was widely accepted in the older anthrax toxin literature that LF and EF are serologically distinct; no cross-reactivity was detected with polyclonal antibodies in immunodiffusion, and this was confirmed with purified components (Quinn *et al.*, 1988). However, once monoclonal antibodies to LF and EF were obtained, several were found that cross-react. In a set of 61 monoclonal antibodies to LF, three cross-reacted with EF (Little *et al.*, 1990). Eight of the LF monoclones neutralized lethal toxin in either *in vivo* or *in vitro* tests, and six of these were shown to do so by preventing LF binding to PA63. Surprisingly, only two of the six that blocked LF binding cross-reacted with EF. This suggests that the sequences shared by LF and EF and presumably involved in binding to PA63 may have

been under selective pressure to become antigenically distinct so that an immune response to one does not effectively neutralize the other.

### *LF metalloprotease activity*

Discovery by the author and his collaborators that LF cleaves MEKs grew out of parallel and independent efforts to identify additional cells sensitive to the lethal toxin and to find better tools for modulating cell signaling pathways. The author tested the cytotoxicity of lethal toxin on 60 human tumor cell lines that constitute a panel used to screen for anti-neoplastic agents (Weinstein *et al.*, 1997). The growth of some cell lines was inhibited, but no cells were lysed in the dramatic manner of mouse macrophages. Several years later, the database was queried by Duesbery and colleagues to find drugs having activity like that of PD98059, which was being used to specifically inhibit the mitogen activated protein kinase kinase 1, also designated MEK1. Of the approximately 60,000 drugs that had been tested against this panel of cells, LF (administered with PA) was the one most similar in action to PD98059. This led to experiments that resulted in the discovery that LF blocks the MAPK pathway and does so by cleaving seven amino acids from the N-terminus of MEK1 (Duesbery *et al.*, 1998). Independent work using a yeast two-hybrid screen for proteins that bind to LF led to the same conclusions (Pellizzari *et al.*, 2000). It was subsequently found that six of the seven MEK family members are cleaved by LF (Vitale *et al.*, 2000), and several are cleaved in two locations (Figure 18.5). Comparison of the sequences at the cleavage sites suggests that LF requires an amino acid sequence motif that can be represented as ++++nHn↓H, where "+" represented Arg or Lys, "n" represents any amino acid, "H" represents hydrophobic amino acids, and "↓" shows the site of cleavage. However, the specificity of LF is also determined by regions in the MEK substrates that are distant from the bond cleaved, as indicated by the ability of MEK1 lacking the N-terminal 30-amino acid cleavage site to bind to LF in the yeast two hybrid system (Vitale *et al.*, 2000). Furthermore, mutation of a region in the C-terminal kinase domain of MEK1 made it resistant to LF cleavage (Chopra *et al.*, 2003). These characteristics mimic those of the clostridial neurotoxins, which also have a highly restricted set of substrates that share specificity determinants distant from the actual cleavage site.

LF protease activity can be inhibited by various nonspecific metalloprotease inhibitors, such as zinc chelating agents. Certain agents that protect mouse macrophages from lethal toxin, including bestatin, aromatic amino acid amides, and hydroxamates (Klimpel *et al.*, 1994; Menard *et al.*, 1996b) may block protease activity, although they could also act on downstream cellular processes involved in cell death. Peptide substrates and inhibitors based on the MEK cleavage sequences have been used to assay LF protease activity and to screen for inhibitors (Cummings *et al.*, 2002; Tonello *et al.*, 2002). High throughput screening and structure-based design is being used to identify small molecule protease inhibitors for treatment of anthrax infections

**FIGURE 18.5** Sequences cleaved by LF. The N-terminal sequences of mitogen-activated protein kinase kinases are shown. The arrow indicates the bond cleaved by LF.

Sites of cleavage by LF within MEK proteins

| Substrate protein | Residue numbers relative to cleavage site | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 6 | 5 | 4 | 3 | 2 | 1 | ⇓ | 1' | 2' | 3' |
| MEK1 | P | K | K | K | P | T | P | | I | Q | L |
| MEK2 | R | R | K | P | V | L | P | | A | L | T |
| MKK3b | K | R | K | K | D | L | R | | I | S | C |
| MKK6b | K | R | N | P | G | L | K | | I | P | K |
| MKK4 | G | K | R | K | A | L | K | | L | N | F |
| MKK4 | P | F | K | S | T | A | R | | F | T | L |
| MKK7β | R | P | R | P | T | L | Q | | L | P | L |
| MKK7β | R | P | R | H | M | L | G | | L | P | S |
| consensus | + | + | + | + | | **H** | | | **H** | | |

Data is from Vitale *et al.* (Vitale, Bernardi, *et al.*, 2000). MKK4 and MKK7β are cleaved at two positions, and sequences are shown for both. "+" and "H" indicate positively charged and hydrophobic residues, respectively.

(Turk *et al.*, 2004; Panchal *et al.*, 2004). It can be anticipated that large pharmaceutical manufacturers may use these leads to develop effective therapeutics, provided that adequate economic incentives are established.

### *LF fusion proteins as vaccines and therapeutics*

As noted above, the similarity of the N-terminal regions of LF and EF implicated this region as the PA-binding domain. The fact that two unrelated catalytic domains could be delivered to the cytosol by linkage to the N-terminal LF/EF domain suggested that this offered a flexible cytosolic delivery system. This hypothesis was confirmed by demonstrating the potency of fusion proteins containing LFn (aa 1–254) and the catalytic domains of other toxins (Arora *et al.*, 1992; Arora and Leppla, 1993; Arora and Leppla, 1994; Milne *et al.*, 1995).

The LFn fusion system has been used by a number of investigators to deliver polypeptides and proteins to the cytosol so as to stimulate cellular immunity, mimicking the generation of epitopes that occurs from degradation of proteins of intracellular pathogens. Delivery of peptide epitopes known to be presented on MHC class I molecules sensitized antigen presenting cells to lysis by specific cytotoxic T cells (Goletz *et al.*, 1997a) and induced cytotoxic T cell responses in animals (Ballard *et al.*, 1996; Lu *et al.*, 2000). Immunization with only 300 fmol (10 ng) of an LF fusion to a well-studied T-cell epitope of listeriolysin protected mice from infection.

Several types of controls showed that successful presentation of the epitope depended on active PA. When the LF fusion protein contained HIV gp120, proteasome activity was needed to process the peptide for presentation (Goletz *et al.*, 1997b). Of several bacterial toxins that have been tested for presentation of CTL epitopes, the anthrax toxin system appears to offer advantages (Goletz *et al.*, 1997b), in particular its efficiency, ability to deliver large polypeptides (Goletz *et al.*, 1997b; Lu *et al.*, 2000), the flexibility in design of LF fusions, and their ease of preparation.

LF fusion proteins have also proven effective for specifically targeting tumor cells *in vitro* and *in vivo*. High specificity for tumor cells has been achieved by replacing the furin cleavage site in PA with a sequence recognized by urokinase plasminogen activator, a protease that is overexpressed on the surface of most types of tumor cells (Liu *et al.*, 2001). In combination with an LF fusion protein to *Pseudomonas* exotoxin A domain III (LFn-PEIII), this agent greatly reduced the sizes of three diverse types of mouse tumors and in some cases led to a complete cure (Liu *et al.*, 2003).

## TOXIN ACTION ON CELLS AND ANIMALS

The anthrax toxins interact to deliver the catalytic components LF and EF to the cytosol of target cells. The role of PA is to cause binding of LF and EF to the cell surface, so that they will be internalized by endocytosis, and to provide a membrane channel for their translocation from endosomes to the cytosol. The effect of EF delivery to the cytosol is predictable from its adenylate cyclase activity and the well-known effects of cAMP and of other toxins that elevate cytosolic cAMP concentrations. LF cleaves MEKs and possibly other unidentified cytosolic proteins. This initiates processes that lead to the lysis of certain mouse macrophages and to the death of animals.

### Cell specificity and receptors

Nearly all types of eucaryotic cells possess receptors for PA. Specific binding of radiolabeled PA to cells is easily demonstrated because more than 80% of total binding can be blocked by addition of non-radioactive PA. Binding can also be demonstrated in fluorescent cell sorters using fluorescent secondary antibodies, whereas the author's lab finds that use of biotin-labeled PA and streptavidin-phycoerythrin is most effective. Quantitative analysis of binding data shows that cells possess a single class of high affinity receptors, having association constants of approximately 1 nM (Singh *et al.*, 1990; Escuyer and Collier, 1991). Different cell types have between 5,000 and 50,000 receptors. Cells with about 50,000 receptors include the L-6 rat myoblast and the human melanoma cell line LOX IMVI. The modest number of receptors, their sensitivity to trypsin, the low non-specific binding, and the linear binding curves suggested that the receptor was a single cell surface protein.

CHO cell mutants lacking functional PA receptors were obtained by selection of mutagenized populations with PA and fusion proteins containing LFn and the catalytic domains of ADP-ribosylating toxins. No cross-resistance was found to other toxins, and all mutants mapped to the same complementation group (Liu and Leppla, 2002). This proved that the receptor gene in CHO cells was functionally hemizygous (i.e., single functional allele). CHO cells of this type provided the basis for an expression cloning approach that successfully identified the receptor (Bradley *et al.*, 2001). A retroviral expression library transfected into receptor-deficient CHO cells was sorted repeatedly after fluorescent labeled for cells having enhanced receptor expression. The clone obtained contained a

cDNA that could restore PA binding to cells. This cDNA, termed ATR (anthrax toxin receptor), was recognized as matching a recently described gene denoted tumor endothelial marker 8 (TEM8), which had been isolated based on its high expression in endothelial cells of colorectal cancer tissues (St Croix *et al.*, 2000). Subsequently, a closely related gene designated capillary morphogenesis gene 2 (CMG2) was also shown to encode a functional receptor (Scobie *et al.*, 2003). Preexisting information from mRNA expression databases suggested that CMG2 is a widely expressed gene, whereas studies on TEM8 indicated that its expression is confined to tumor tissues (Nanda and St Croix, 2004). Nevertheless, there remains uncertainty about which of these receptors is expressed in widely used cultured cells, and more importantly, in the tissues of animals and humans that become infected by *B. anthracis*.

CMG2 and TEM8 contain a domain like the von Willebrand or I (inserted) domain that is characteristic of many integrins. The I domain contains a metal ion dependent adhesion site (MIDAS) that is essential for binding to the extracellular matrix molecules, such as collagens that are the recognized ligands for these integrins. Mutagenesis of this site showed it was required for PA binding (Bradley *et al.*, 2003). Mutagenesis of PA showed that the D683 side chain, previously proven to be important for PA function, was a strong candidate for a residue that would interact with the metal ion bound at the MIDAS of the I domain. Purification of recombinant forms of the extracellular domains of TEM8 and CMG2 allowed detailed study of their interaction with PA. A structure was obtained for the extracellular domain of CMG2 (Lacy *et al.*, 2004b) that was consistent with other I domain proteins. PA bound with very high affinity, Kd = 170 pM, to the recombinant CMG2 (Wigelsworth *et al.*, 2004). Most importantly, structures were obtained for the complex of PA with CMG2 (Santelli *et al.*, 2004; Lacy *et al.*, 2004a). These structures demonstrated, somewhat surprisingly, that PA interacts with CMG2 not only through the surface of domain 4 but also via regions on domain 2. The large surface area of the interaction, 1300 $Å^2$, explains the high affinity of PA binding. The other important conclusion from the structure was that the interaction with receptor is predicted to prevent the movement of the PA domain 2 loop that occurs during the acid-induced insertion into cellular membranes. This brace or clamping action of the receptor imposes a requirement that the toxin reach a late, more highly acidic endocytic compartment to undergo prepore to pore conversion. This is consistent with new data on intracellular trafficking that is discussed below.

## Proteolytic activation of PA by furin and other proteases

PA bound to cellular receptors must be activated by proteolytic cleavage at aa 167 to acquire the ability to oligomerize and to bind LF and EF (discussed above). The cellular enzyme that most rapidly activates PA is furin (Klimpel *et al.*, 1992), because furin-deficient CHO and LoVo cells are much less sensitive than parental cells to the combination of PA and LFn-PEIII (Gordon *et al.*, 1995). However, the cleavage site, RKKR, has more basic residues than the minimum furin recognition sequence RxxR, and appears to be susceptible to other cellular proteases. Thus, furin-deficient cells retain some sensitivity to wild-type PA but are totally resistant to PA mutants with a RAAR sequence. It has been found for several other toxins and viruses that furin is the dominant activating enzyme, but that other cellular proteases can also perform the activation, although less efficiently. It is also of interest that urokinase plasminogen activator, a protease abundant on tumor cells, can very efficiently activate PA mutants having appropriate target sequences substituted at aa 164. This implies that receptor-bound PA is either mobile in the lipid membrane or that any localization into membrane domains does not prevent contact with certain cell-surface proteases.

## Toxin internalization and translocation across membranes

LF and EF bind with high affinity to the proteolytically activated, cell-surface bound PA heptamer. Association constants of 150–700 pM, determined on cells with radiolabeled LF and EF or by competitive Schild plots, agree well with a value of 1,000 pM obtained for purified heptamer by surface plasmon resonance (Novak *et al.*, 1992; Elliott, Mogridge et al., 2000).

The complex of PA63 heptamer and LF and EF enters cells by receptor-mediated endocytosis and reaches an acidic vesicular compartment, because toxicity is blocked by pharmacological agents that prevent acidification, including amines (Friedlander, 1986; Gordon *et al.*, 1988) and bafilomycin, a specific inhibitor of proton pumps (Menard *et al.*, 1996a). Although the normal trafficking of the TEM8 and CMG2 receptors is not yet characterized, it has been possible to determine the behavior of the receptor-bound PA. When PA cannot be cleaved at the furin site, it remains at the cell surface for extended times, indicating that the receptors are not normally internalized in a specific manner, but only at the default rate of all membrane proteins (Beauregard *et al.*, 2000; Liu and Leppla, 2002; Abrami *et al.*, 2003). However, following protease cleavage, PA63 rapidly

associates into the heptamer, and this is localized into specialized, cholesterol-rich membrane domains called *lipid rafts*, which are then rapidly internalized (Abrami *et al.*, 2003). Somewhat surprisingly, the internalization is dependent on clathrin, which is unusual for lipid raft-dependent endocytic processes (Abrami *et al.*, 2004).

In an intracellular compartment, a more acidic environment is believed to trigger a major rearrangement of the PA heptamer that inserts the amphipathic loop sequences into the lipid bilayer. As noted above, this probably involves insertion of aa 275 to 352 as an extended β barrel, analogous to that in the structure determined for staphylococcal alpha-toxin. This event can be mimicked at the cell surface, providing a more convenient system in which to study the process. Experimentally, cells having LF associated with surface-bound PA heptamer are briefly treated with pH 5.0 buffer (Milne and Collier, 1993; Singh *et al.*, 1994). Acidification leads to toxicity, in a process that can no longer be blocked by agents that inhibit acidification of cellular compartments (Friedlander, 1986; Gordon *et al.*, 1988). Insertion to produce a plasma membrane channel can also be detected by measuring the efflux of radioactive $Rb^+$ or $Na^+$ previously loaded into the cells. PA was also shown to form ion-conductive channels in artificial lipid membranes containing phospholipids (Blaustein *et al.*, 1989; Finkelstein, 1994). Insertion requires lowering the pH to 6.5. The electrical properties of the channels are pH and voltage dependent, and the channel can be blocked by large, quaternary ammonium ions. The passage of smaller quaternary ammonium ions leads to an estimate that the lumen of the channel is approximately 11 Å at its most narrow point.

All members of the PA family have similar amphipathic β hairpin forming sequences that are likely to produce membrane-inserted β barrels (Figure 18.6). The pair of Phe residues in PA, Phe313-Phe314, previously shown to be essential for PA activity, are at the turn of the loop. It is interesting that all members of the family except PA have a His-Ser or His-Thr sequence following the β hairpin. Protonation of this or other His residues may be involved in the acid-dependent membrane insertion (Petosa *et al.*, 1997; Santelli *et al.*, 2004).

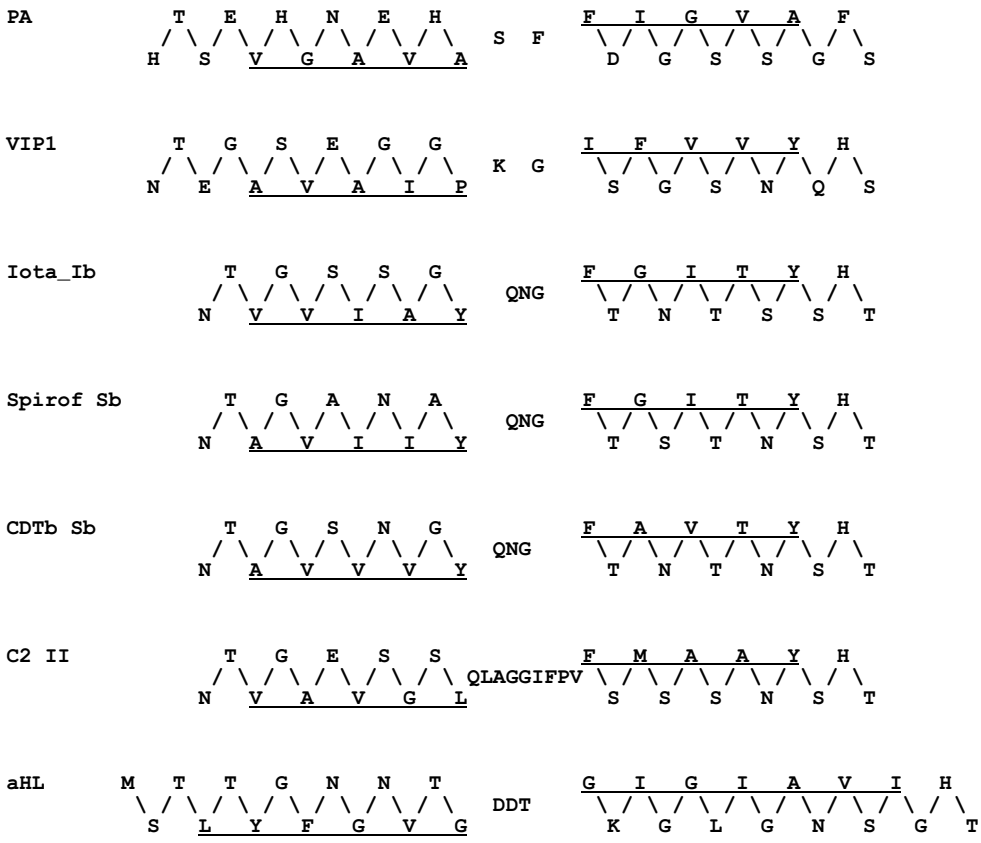



**FIGURE 18.6** Amphipathic sequences of PA family members. Sequences from PA family members that are known or proposed to form amphipathic hairpins that assemble into membrane-spanning barrel structures. Hydrophobic residues (underlined) will face the lipid bilayer and the alternating hydrophilic residues will face the lumen of the channel. Residues in the central region (SF in the case of PA) form the bend of the hairpin located on the cytosolic side of the membrane.

Prior to membrane insertion, the PA63 heptamer that assembles on the cell surface is viewed as a prepore. Experimentally, it is characterized as being quite stable, presumably being equivalent to the PA63 oligomer that is produced by trypsin treatment and MonoQ chromatography, as described above. However, this form does not survive heating in SDS, as is typically done when preparing samples for SDS gel electrophoresis (Leppla, 1991b; Milne *et al.*, 1994; Singh *et al.*, 1994; Miller *et al.*, 1999). Transition to an active pore, following acid-induced insertion into the membrane, creates a more stable structure that survives heating in SDS. The heat-stable oligomer can be produced by acid treatment of soluble PA63 or acid shock of cells having surface-bound PA63 heptamer, but is formed most efficiently by allowing normal cellular internalization to an endocytic compartment (Liu and Leppla, 2002).

Early studies based on similarities to diphtheria toxin trafficking suggested that LF and EF were translocated from early endosomes to the cytosol. However, more recent work suggests that translocation occurs more slowly, suggestive of release from a later endosomal compartment (Menard *et al.*, 1996a; Abrami *et al.*, 2004). In particular, a requirement for pH values approaching 5.0, and the structural information noted above showing that the CMG2 receptor clamps PA domains 2 and 4 to limit membrane insertion, suggested that the acidity of early endosomes (typically pH 6.0) is not sufficient to induce membrane insertion. A detailed trafficking study then showed that the SDS-resistant PA heptamer, the result of membrane insertion, was present in isolated early endosomes, but that LF translocation to the cytosol required transport to a later endosomal compartment. A model was suggested in which LF and EF are delivered into intraluminal vesicles, which later fuse back to late endosomal membranes to release their contents to the cytosol (Abrami *et al.*, 2004).

One of the least understood steps in anthrax toxin trafficking is the transfer of LF and EF across endosomal membranes. Questions examined have been what part of LF initiates transfer, whether translocation is through the lumen of the heptamer, whether the LF must unfold, and what drives the translocation. It was originally suggested that translocation began at the N-terminus, but LF fusion proteins having polypeptides attached at either end of LFn are both delivered (Arora and Leppla, 1994; Milne *et al.*, 1995; Ballard *et al.*, 1996). While this suggests that an internal sequence having the form of a hairpin loop must initiate translocation, additional evidence from artificial lipid membrane studies argued for initiation at the N-terminus (Zhang *et al.*, 2004a). Conceptually, it has seemed easier to imagine that translocation occurred via the 11 Å lumen of the PA63 heptamer, although this does then require partial unfolding of the transiting polypeptides. Direct evidence that LF and EF are translocated through the lumen of the heptamer comes from studies in artificial lipid membranes (Zhang *et al.*, 2004b). LFn enters and plugs the channel at low transmembrane potentials but is driven through the channel at higher voltages. The force that might drive the entire polypeptide through the lumen is less well understood, because its charge distribution does not predict a simple electrostatically-driven process (Zhang *et al.*, 2004b). One model holds that refolding of the protein on the cytosolic side provides the force to prevent reverse movement. In the case of diphtheria toxin, chaperones have been identified that assist the refolding, and thereby provide a ratchet device force to promote unidirectional membrane transit (Ratts *et al.*, 2003), but parallel studies suggested that the same chaperones are not required for LF transit (Haug *et al.*, 2003). Finally, to confirm that unfolding is required, LF fusion proteins were constructed with added, internal disulfide bonds. The inability of these to be delivered from the cell surface in the acid shock protocol supports the view that unfolding is required (Wesche *et al.*, 1998). A requirement for unfolding limits the types of polypeptides that can be delivered as fusions to LFn in the vaccine and therapeutic applications discussed above.

## Cytosolic actions and cellular effects of EF and LF

### *ET action on cells*

Edema toxin treatment of nearly all cell types causes an increase in cAMP. This provides additional evidence that most cell types possess receptors and a protease able to activate PA. The maximum levels of cAMP reached in treated cells differ greatly, from levels that barely exceed the basal concentration of about 2 μmol cAMP $mg^{-1}$ of cell protein to levels of 2000 μ mol cAMP $mg^{-1}$ (Leppla, 1984; Gordon *et al.*, 1988; Gordon *et al.*, 1989). The consequences of elevated cAMP levels have been studied for decades, following the discovery that cAMP acts as a signaling molecule or "second messenger" to activate cAMP-dependent protein kinase (protein kinase A, PKA).

Full activation of PKA requires only a modest elevation of cAMP concentration above basal levels. Activated PKA phosphorylates a number of proteins and alters many cellular processes, the full details of which cannot be described here. Very high cAMP concentrations are not lethal to cultured cells, so toxin-treated cells can recover after removal of toxin. Unlike with cholera toxin, the effects of edema toxin are rap-

idly reversed, apparently because EF is unstable in the cytosol (Leppla, 1982), with a half life of less than two hours.

There are also suggestions that cAMP produced by EF could act through PKA-independent processes. Large increases in cAMP come at the expense of its precursor, ATP, which theoretically could be depleted by >50%, thereby impacting basic cellular energy-dependent processes. Furthermore, cAMP-binding domains have been identified in proteins other than PKA (Chin *et al.*, 2002), including proteins that modulate the same MEK pathways that are targeted by LF. It also must be considered that the amounts and effects of EF-generated cAMP will vary depending on its location within various cytosolic compartments (Tasken and Aandahl, 2004).

Relatively little work has examined the consequences of ET action on cultured cells. ET is believed to play an important role early in an anthrax infection because elevated cAMP concentrations incapacitate phagocytic cells (Confer and Eaton, 1982). Thus, human polymorphonuclear cells treated with purified edema toxin are unable to phagocytose opsonized *B. anthracis* (O'Brien *et al.*, 1985). Edema toxin also has profound effects on chemotaxis and on endotoxin priming of polymorphonuclear cells (Wright *et al.*, 1988). Human monocytes treated with edema toxin produced more IL-6 and were blocked in their endotoxin-induced TNF production (Hoover *et al.*, 1994). Recent studies demonstrated that ET induces calcium effluxes from cultured polarized epithelial cells (Beauregard *et al.*, 1999) and induces a rapid calcium influx in several other cell types (Kumar *et al.*, 2002). In the latter study, the calcium influx occurred prior to the large increases in cAMP concentration and was required for this to occur. It is possible that a small initial increase in cAMP causes calcium channels to open, and the resulting increased cytosolic calcium concentration enhances EF catalytic activity. Regardless of any differences in cellular response to ET, it is clear that the high levels of cAMP produced by EF will have profound effects on nearly all types of cells.

### *LF action on cells*

The known enzymatic action of LF, cleavage and inactivation of MEKs, will effectively shut down several key MEK signaling pathways, but this would not be expected to rapidly kill cells. However, one cell type does respond in an acute manner to lethal toxin. Certain mouse macrophages (e.g., those from BALB/cJ mice) treated with lethal toxin lyse in 90–120 minutes (Friedlander, 1986). Both resident and elicited primary macrophages are susceptible, as are certain macrophage-like cell lines, including J774A.1 and RAW 264.7. However, macrophages from many inbred mouse strains (e.g., C57Bl/6J) are highly resistant to lysis, even though LT enters these cells and cleaves MEKs. This strong phenotypic difference allowed mapping of the genetic locus controlling susceptibility to a region of mouse chromosome 11 that contains the gene encoding Kif1c, a kinesin-like motor protein (Watters *et al.*, 2001). Later studies suggested that the region near the Kif1c gene contains three genes ("quantitative trait loci") that control susceptibility of mice to LT (McAllister *et al.*, 2003). However, no molecular explanation has yet been provided as to how Kif1C or the unidentified neighboring genes might control LT action. If Kif1c plays a role, it may be through regulating the subcellular location of LF or its key substrates. It may also be relevant that LF potency depends on resistance to degradation in the cytosol, which for LF has been shown to involve the proteosome (Falnes and Olsnes, 1998).

Although the rapid *in vitro* lysis of certain mouse macrophages is dramatic, more relevant to events during an anthrax infection is the observation that macrophages stimulated with bacterial cell products such as poly-glutamic acid become susceptible to LT and are killed in a process that has features more characteristic of necrosis than apoptosis. Susceptibility was found in mouse and human macrophages, regardless of the identity of the loci on chromosome 11 (Park *et al.*, 2002; Kim *et al.*, 2002). In parallel incubations lacking LT, some cell death was produced in the activated macrophages by combining inhibitors of all three MEK pathways, suggesting that the LT-induced death could be due to simultaneous inhibition of the same pathways.

Much effort has been made to understand the events leading to the rapid macrophage lysis in the inherently LT-susceptible macrophages (i.e., BALB/cJ). The earliest measured changes in LT-treated macrophages are increases in $K^+$ and $Rb^+$ ion fluxes at 45 minutes, decreases in ATP concentrations and release of superoxide at 60 minutes, and changes in morphology at 75 minutes, which progress to lysis at 90 minutes (Bhatnagar *et al.*, 1989; Hanna *et al.*, 1992; Hanna *et al.*, 1994). The changes occurring after 60 minutes can be delayed by removal of extracellular calcium and by osmotic stabilizers, suggesting that membrane integrity is compromised. Reducing agents also protect mouse macrophages from lethal toxin (Hanna *et al.*, 1994), indicating that the oxidative burst is required for cell lysis. The inability to connect cleavage of MEKs to this rapid lysis has led to the suggestion that LF may cleave other proteins that are critical to macrophage homeostatis, but searches have not identified additional LF substrates.

LT has a more limited effect on other cells studied *in vitro*. Certain cultured cells are inhibited in growth if plated at low cell densities and treated with lethal toxin for 3–7 days. A screen of the 60 tumor cell lines available in the National Cancer Institute drug screening program identified melanoma cells as especially sensitive to LT (Koo *et al.*, 2002), an effect shown to involve apoptosis. This finding is the basis for ongoing development of LT as a cancer treatment (Frankel *et al.*, 2003). Endothelial cells were reported to be killed by LT in an apoptotic process, although the fraction of cells dying after several days was variable (Kirby, 2004). Dendritic cells were found to be targeted by LT with a resultant failure to function in several aspects of the host immune responses (Agrawal *et al.*, 2003). LT has also been found to block transcriptional activation by the glucocorticoid receptor (Webster *et al.*, 2003).

## Actions of the lethal and edema toxins in animals

Anthrax toxin was first recognized by noting the edema caused by intradermal injection of bacterial culture supernatant or plasma of infected animals (Smith *et al.*, 1955). Fractionation showed that this effect was due to the mixture of components now termed *edema toxin*. Subsequent studies showed the supernatant also contained the separate lethal toxin. Unfortunately, those early studies employed toxin preparations of uncertain purity, and on reexamination provide few insights into the cellular systems that are damaged by toxin *in vivo*. However, it is accepted that the effects of lethal toxin administration closely resemble those observed in animals dying from infection with virulent *B. anthracis*. It is for this reason that the authors and others have resumed studies on the pathophysiological effects of the toxins in experimental animals. One goal of such work is to provide guidance in design of prophylactic and therapeutic agents for infected humans.

The best-characterized *in vivo* action of the lethal toxin is its rapid toxicity in rats, where it causes overwhelming pulmonary edema and death in as little as 38 minutes (Haines *et al.*, 1965; Ezzell *et al.*, 1984). The Fischer 344 rat is particularly sensitive to the toxin. The edematous fluid in the lungs of the rats has the protein composition of serum, implying a cytotoxic effect that leads to failure of the endothelial barrier, rather than stimulation of fluid secretion. Recent studies in which LT was infused into Sprague-Dawley rats to mimic toxin accumulation during an infection showed a progressive decline in cardiovascular function preceding a shock-like death. Unlike endotoxin-induced shock, there were no associated cytokine responses (Cui *et al.*, 2004). Although the causes of this rapid death in rats are not understood, the rapid lethality provides a convenient *in vivo* bioassay for evaluation of therapeutics such as neutralizing antibodies, small molecule inhibitors blocking LF catalysis, and others. An advantage of this assay is that its short duration lessens concerns about the residence time and tissue distribution of the candidate therapeutic agents.

Studies of LT action in mice were conducted to extend the finding that mouse macrophages are rapidly lysed and that the susceptibility of mice appeared to correlate with the LT susceptibility of their macrophages. LT was reported to induce IL-1 and TNF in macrophages, and blocking the action of these cytokines protected mice from death by injected LT (Hanna *et al.*, 1993). Furthermore, depletion of macrophages made mice resistant to the lethal action of LT. These studies led to the hypothesis (Hanna, 1998) that macrophages are induced to synthesize and then release unphysiologically high concentrations of cytokines, which then cause a shock-like response. However, these finding have not been confirmed in other laboratories (Erwin *et al.*, 2001) and results noted above and discussed below argue against a key role for cytokines in lethality. In fact, the general finding is that LT treatment profoundly suppresses host immune responses and limits inflammatory responses.

Extensive studies of the action of LT in a number of inbred mouse strains has led to the conclusion that death is due to a circulatory collapse and hypoxia in key organs, particularly bone marrow, liver, and spleen (Moayeri *et al.*, 2003; Moayeri and Leppla, 2004). Hypoxia, liver necrosis, and pleural edema were noted. There was no clear correlation within a set of inbred strains between sensitivity of the mice and of their macrophages to LT, arguing against a key role of macrophage lysis in lethality. Thus, BALB/cJ mice (having LT-sensitive macrophages) died more rapidly and showed a rapid but transient cytokine burst, whereas C57Bl/6J mice (having LT-resistant macrophages) were also killed, although more slowly and with no cytokine burst. A clear test of the contribution of macrophages came from comparing two nearly isogenic BALB/cJ mouse strains differing only in the Kif1c region on chromosome 11. It was found that the LT-induced lysis of macrophages in the mice having sensitive macrophages was needed to obtain the transient cytokine burst, suggesting this explained their slightly increased lethality (Moayeri *et al.*, 2004). It was also shown that the relative sensitivities of many other inbred mouse strains to LT depends on genetic factors in addition to those in the Kif1c region.

In this laboratory, studies have also begun on the action of purified ET in animals. These studies became possible after improved methods for EF production

were developed (Soelaiman *et al.*, 2003). Somewhat surprisingly, ET rapidly killed mice and did so at doses lower than those required for LT (Firoved *et al.*, submitted, 2004). Extensive effusions in the gastrointestinal tract and hemorrhage of the ileum and adrenal glands were observed. Changes in nearly every biochemical parameter measured indicated that ET damages many organs and tissues and probably kills by inducing multi-organ failure. It will now be useful to extend these studies to characterize any synergistic effects of ET and LF in animals.

## CONCLUSION

It is clear from the data presented above that the anthrax toxins play a dominant role in the pathogenesis of anthrax infections. Strains unable to produce the toxin are avirulent, and animals are protected against infection only if they possess antibodies to the toxin. Other bacterial pathogens depend on protein exotoxins for their virulence, but in most cases the toxins have a less complex design. A unique feature of the anthrax toxins is the segregation of the receptor binding and translocation functions on a protein separate from the effector function. This binary design is clearly an effective one, because it is utilized by at least five other toxins produced by Gram-positive pathogens.

Rapid progress has occurred in recent years in understanding the interaction of the anthrax toxin proteins with cells. The toxin receptor has been identified as a blood vessel protein, which may explain the increasing evidence that LT targets the vascular system. The role of furin in proteolytic activation of PA has been detailed. The structure and function of the protein-conducting heptameric PA channel is now one of the best characterized membrane channel proteins. The catalytic activity of LF has been identified and its role in targeting MEK pathways characterized. There remain important questions about how the edema and lethal toxins act in cells and animals to cause the pathology associated with anthrax infection. We can anticipate important progress in connecting the initial catalytic steps, in particular the proteolytic action of LF, to the subsequent pathologic effects in cells and tissues.

The recognition of the need for improved anthrax vaccines has led to a great deal of study on ways to induce antibodies to PA, and to a lesser extent, LF and other potential immunogens. In fact, the great diversity of vaccine carriers being used to deliver PA will allow a useful comparison of their relative efficacies that will have a wide impact on design of other vaccines. Finally, the greatly increased knowledge of the toxin's structure and function has provided a firm basis for the design of a diverse set of therapeutic agents designed to protect and treat anthrax infections. Increased government support for anthrax vaccines and therapeutics assures that a number of effective agents will become available in the next few years.

## REFERENCES


Abrami, L., Lindsay, M., Parton, R.G., Leppla, S.H. and van der Goot, F.G. (2004). Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. *J. Cell Biol.* **166,** 645–651.

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003). Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J. Cell Biol.* **160,** 321–328.

Agrawal, A., Lingappa, J., Leppla, S.H., Agrawal, S., Jabbar, A., Quinn, C. and Pulendran, B. (2003). Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* **424,** 329–334.

Ahuja, N., Kumar, P. and Bhatnagar, R. (2004). The adenylate cyclase toxins. *Crit. Rev. Microbiol.* **30,** 187–196.

Arora, N., Klimpel, K.R., Singh, Y. and Leppla, S.H. (1992). Fusions of anthrax toxin lethal factor to the ADP-ribosylation domain of *Pseudomonas* exotoxin A are potent cytotoxins, which are translocated to the cytosol of mammalian cells. *J. Biol. Chem.* **267,** 15542–15548.

Arora, N. and Leppla, S.H. (1993). Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem.* **268,** 3334–3341.

Arora, N. and Leppla, S.H. (1994). Fusions of anthrax toxin lethal factor with Shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun.* **62,** 4955–4961.

Ascenzi, P., Visca, P., Ippolito, G., Spallarossa, A., Bolognesi, M. and Montecucco, C. (2002). Anthrax toxin: a tripartite lethal combination. *FEBS Lett.* **531,** 384–388.

Ballard, J.D., Collier, R.J. and Starnbach, M.N. (1996). Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope *in vivo. Proc. Natl. Acad. Sci. U. S. A.* **93,** 12531–12534.

Barth, H., Aktories, K., Popoff, M.R. and Stiles, B.G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol. Mol. Biol. Rev.* **68,** 373–402.

Bartkus, J.M. and Leppla, S.H. (1989). Transcriptional regulation of the protective antigen gene of *Bacillus anthracis. Infect. Immun.* **57,** 2295–2300.

Battisti, L., Green, B.D. and Thorne, C.B. (1985). Mating system for transfer of plasmids among *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis. J. Bacteriol.* **162,** 543–550.

Beauregard, K.E., Collier, R.J. and Swanson, J.A. (2000). Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization. *Cell Microbiol.* **2,** 251–258.

Beauregard, K.E., Wimer-Mackin, S., Collier, R.J. and Lencer, W.I. (1999). Anthrax toxin entry into polarized epithelial cells. *Infect. Immun.* **67,** 3026–3030.

Benson, E.L., Huynh, P.D., Finkelstein, A. and Collier, R.J. (1998). Identification of residues lining the anthrax protective antigen channel. *Biochemistry* **37,** 3941–3948.

Bhatnagar, R., Singh, Y., Leppla, S.H. and Friedlander, A.M. (1989). Calcium is required for the expression of anthrax lethal toxin

activity in the macrophagelike cell line J774A.1. *Infect. Immun.* **57,** 2107–2114.

Blaustein, R.O., Koehler, T.M., Collier, R.J. and Finkelstein, A. (1989). Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2209–2213.

Bourgogne, A., Drysdale, M., Hilsenbeck, S.G., Peterson, S.N. and Koehler, T.M. (2003). Global effects of virulence gene regulators in a *Bacillus anthracis* strain with both virulence plasmids. *Infect. Immun.* **71,** 2736–2743.

Bradley, K.A., Mogridge, J., Mourez, M., Collier, R.J. and Young, J.A. (2001). Identification of the cellular receptor for anthrax toxin. *Nature* **414,** 225–229.

Bradley, K.A., Mogridge, J., Rainey, G.J., Batty, S. and Young, J.A. (2003). Binding of anthrax toxin to its receptor is similar to alpha integrin-ligand interactions. *J. Biol. Chem.* **278,** 49342–49347.

Brossier, F., Levy, M., Landier, A., Lafaye, P. and Mock, M. (2004). Functional analysis of *Bacillus anthracis* protective antigen by using neutralizing monoclonal antibodies. *Infect. Immun.* **72,** 6313–6317.

Brossier, F., Sirard, J.C., Guidi-Rontani, C., Duflot, E. and Mock, M. (1999). Functional analysis of the carboxy-terminal domain of *Bacillus anthracis* protective antigen. *Infect. Immun.* **67,** 964–967.

Cataldi, A., Labruyere, E. and Mock, M. (1990). Construction and characterization of a protective antigen-deficient *Bacillus anthracis* strain. *Mol. Microbiol.* **4,** 1111–1117.

Chauhan, V. and Bhatnagar, R. (2002). Identification of amino acid residues of anthrax protective antigen involved in binding with lethal factor. *Infect. Immun.* **70,** 4477–4484.

Chin, K.V., Yang, W.L., Ravatn, R., Kita, T., Reitman, E., Vettori, D., Cvijic, M.E., Shin, M. and Iacono, L. (2002). Reinventing the wheel of cyclic AMP: novel mechanisms of cAMP signaling. *Ann. N. Y. Acad. Sci.* **968,** 49–64.

Chopra, A.P., Boone, S.A., Liang, X. and Duesbery, N.S. (2003). Anthrax lethal factor proteolysis and inactivation of MAP-kinase-kinase. *J. Biol. Chem.* **278,** 9402–9406.

Collier, R.J. and Young, J.A.T. (2003). Anthrax toxin. *Ann. Rev. Cell Dev. Biol.* **19,** 45–70.

Confer, D.L. and Eaton, J.W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* **217,** 948–950.

Cooksey, B.A., Sampey, G.C., Pierre, J.L., Zhang, X., Karwoski, J.D., Choi, G.H. and Laird, M.W. (2004). Production of biologically active *Bacillus anthracis* edema factor in *Escherichia coli. Biotechnol Prog.* **20,** 1651–1659.

Cui, X., Moayeri, M., Li, Y., Li, X., Haley, M., Fitz, Y., Correa-Araujo, R., Banks, S.M., Leppla, S.H. and Eichacker, P.Q. (2004). Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats. *Am. J. Physiol Regul. Integr. Comp Physiol* **286,** R699–R709.

Cummings, R.T., Salowe, S.P., Cunningham, B.R., Wiltsie, J., Park, Y.W., Sonatore, L.M., Wisniewski, D., Douglas, C.M., Hermes, J.D. and Scolnick, E.M. (2002). A peptide-based fluorescence resonance energy transfer assay for *Bacillus anthracis* lethal factor protease. *Proc. Natl. Acad. Sci. U. S. A* **99,** 6603–6606.

Cunningham, K., Lacy, D.B., Mogridge, J. and Collier, R.J. (2002). Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc. Natl. Acad. Sci. U. S. A* **99,** 7049–7053.

Dai, Z., Sirard, J.C., Mock, M. and Koehler, T.M. (1995). The *atxA* gene product activates transcription of the anthrax toxin genes and is essential for virulence. *Mol. Microbiol.* **16,** 1171–1181.

Dixon, T.C., Meselson, M., Guillemin, J. and Hanna, P.C. (1999). Medical Progress: Anthrax. *N. Engl. J. Med.* **341,** 815–826.

Drum, C.L., Yan, S.Z., Bard, J., Shen, Y.Q., Lu, D., Soelaiman, S., Grabarek, Z., Bohm, A. and Tang, W.J. (2002). Structural basis for the activation of anthrax adenylyl cyclase exotoxin by calmodulin. *Nature* **415,** 396–402.

Drysdale, M., Bourgogne, A., Hilsenbeck, S.G. and Koehler, T.M. (2004). *atxA* controls *Bacillus anthracis* capsule synthesis via *acpA* and a newly discovered regulator, *acpB*. *J. Bacteriol.* **186,** 307–315.

Duesbery, N.S. and Vande Woude, G.F. (1999). Anthrax toxins. *Cell Mol. Life Sci.* **55,** 1599–1609.

Duesbery, N.S. *et al.* (1998). Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280,** 734–737.

Elliott, J.L., Mogridge, J., and Collier, R.J. (2000). A quantitative study of the interactions of *Bacillus anthracis* edema factor and lethal factor with activated protective antigen. *Biochemistry* **39,** 6706–6713.

Erwin, J.L., DaSilva, L.M., Bavari, S., Little, S.F., Friedlander, A.M. and Chanh, T.C. (2001). Macrophage-derived cell lines do not express proinflammatory cytokines after exposure to *Bacillus anthracis* lethal toxin. *Infect. Immun.* **69,** 1175–1177.

Escuyer, V. and Collier, R.J. (1991). Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. *Infect. Immun.* **59,** 3381–3386.

Escuyer, V., Duflot, E., Sezer, O., Danchin, A. and Mock, M. (1988). Structural homology between virulence-associated bacterial adenylate cyclases. *Gene* **71,** 293–298.

Ezzell, J.W., Ivins, B.E. and Leppla, S.H. (1984). Immunoelectrophoretic analysis, toxicity, and kinetics of *in vitro* production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect. Immun.* **45,** 761–767.

Falnes, P.O. and Olsnes, S. (1998). Modulation of the intracellular stability and toxicity of diphtheria toxin through degradation by the N-end rule pathway. *EMBO J.* **17,** 615–625.

Farchaus, J.W., Ribot, W.J., Jendrek, S. and Little, S.F. (1998). Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of *Bacillus anthracis. Appl. Environ. Microbiol.* **64,** 982–991.

Finkelstein, A. (1994). The channel formed in planar lipid bilayers by the protective antigen component of anthrax toxin. *Toxicology* **87,** 29–41.

Firoved *et al.*, submitted, 2004.

Frankel, A.E., Koo, H.M., Leppla, S.H., Duesbery, N.S. and Wounde, G.F. (2003). Novel protein targeted therapy of metastatic melanoma. *Curr. Pharm. Des.* **9,** 2060–2066.

Friedlander, A.M. (1986). Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.* **261,** 7123–7126.

Gill, D.M. (1978). Seven toxin peptides that cross cell membranes. In: *Bacterial Toxins and Cell Membranes* (eds. J. Jeljaszewicz and T. Wadstrom) pp. 291–332. Academic Press, Inc., New York.

Gladstone, G.P. (1946). Immunity to anthrax. Protective antigen present in cell-free culture filtrates. *Brit. J. Exp. Path.* **27,** 349–418.

Gladstone, G.P. (1948). Immunity to anthrax. Production of the cell-free protein antigen in cellophane sacs. *Brit. J. Exp. Path.* **29,** 379.

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullmann, A. and Danchin, A. (1988). The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli. Mol. Microbiol.* **2,** 19–30.

Goletz, T.J., Klimpel, K.R., Arora, N., Leppla, S.H., Keith, J.M. and Berzofsky, J.A. (1997a). Targeting HIV proteins to the major histocompatibility complex class I processing pathway with a novel gp120-anthrax toxin fusion protein. *Proc. Natl. Acad. Sci. U. S. A* **94,** 12059–12064.

Goletz, T.J., Klimpel, K.R., Leppla, S.H., Keith, J.M. and Berzofsky, J.A. (1997b). Delivery of antigens to the MHC class I pathway using bacterial toxins. *Hum. Immunol.* **54,** 129–136.

Gordon, V.M., Benz, R., Fujii, K., Leppla, S.H. and Tweten, R.K. (1997). *Clostridium septicum* alpha-toxin is proteolytically activated by furin. *Infect. Immun.* **65,** 4130–4134.

Gordon, V.M., Klimpel, K.R., Arora, N., Henderson, M.A. and Leppla, S.H. (1995). Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases. *Infect. Immun.* **63,** 82–87.

Gordon, V.M., Leppla, S.H. and Hewlett, E.L. (1988). Inhibitors of receptor-mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infect. Immun.* **56,** 1066–1069.

Gordon, V.M., Young, W.W., Jr., Lechler, S.M., Gray, M.C., Leppla, S.H. and Hewlett, E.L. (1989). Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis*. Different processes for interaction with and entry into target cells. *J. Biol. Chem.* **264,** 14792–14796.

Green, B.D., Battisti, L., Koehler, T.M., Thorne, C.B. and Ivins, B.E. (1985). Demonstration of a capsule plasmid in *Bacillus anthracis*. *Infect. Immun.* **49,** 291–297.

Guo, Q., Shen, Y., Zhukovskaya, N.L., Florian, J. and Tang, W.J. (2004). Structural and kinetic analyses of the interaction of anthrax adenylyl cyclase toxin with reaction products cAMP and pyrophosphate. *J. Biol. Chem.* **279,** 29427–29435.

Gupta, P., Batra, S., Chopra, A.P., Singh, Y. and Bhatnagar, R. (1998). Expression and purification of the recombinant lethal factor of *Bacillus anthracis*. *Infect. Immun.* **66,** 862–865.

Gupta, P., Singh, A., Chauhan, V. and Bhatnagar, R. (2001). Involvement of residues 147VYYEIGK153 in binding of lethal factor to protective antigen of *Bacillus anthracis*. *Biochem. Biophys. Res. Commun.* **280,** 158–163.

Haines, B.W., Klein, F. and Lincoln, R.E. (1965). Quantitative assay for crude anthrax toxins. *J. Bacteriol.* **89,** 74–83.

Hambleton, P., Carman, J.A. and Melling, J. (1984). Anthrax: the disease in relation to vaccines. *Vaccine* **2,** 125–132.

Hanna, P. (1998). Anthrax pathogenesis and host response. *Curr. Top. Microbiol. Immunol.* **225,** 13–35.

Hanna, P.C., Acosta, D. and Collier, R.J. (1993). On the role of macrophages in anthrax. *Proc. Natl. Acad. Sci. U. S. A* **90,** 10198–10201.

Hanna, P.C., Kochi, S. and Collier, R.J. (1992). Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. *Mol. Biol. Cell* **3,** 1269–1277.

Hanna, P.C., Kruskal, B.A., Ezekowitz, R.A., Bloom, B.R. and Collier, R.J. (1994). Role of macrophage oxidative burst in the action of anthrax lethal toxin. *Mol. Med.* **1,** 7–18.

Hanski, E. and Coote, J.G. (1991). *Bordetella pertussis* adenylate cyclase toxin. In: *Sourcebook of Bacterial Protein Toxins* (eds. J. E. Alouf and J. H. Freer), pp. 349–366. Academic Press, London.

Haug, G., Leemhuis, J., Tiemann, D., Meyer, D.K., Aktories, K. and Barth, H. (2003). The host cell chaperone Hsp90 is essential for translocation of the binary *Clostridium botulinum* C2 toxin into the cytosol. *J. Biol. Chem.* **278,** 32266–32274.

Heemskerk, D.D. and Thorne, C.B. (1990). Genetic exchange and transposon mutagenesis in *Bacillus anthracis*. *Salisbury Med. Bull.* **68,** *Spec. suppl.*, 63–67.

Hoffmaster, A.R. *et al.* (2004). Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 8449–8454.

Hoover, D.L., Friedlander, A.M., Rogers, L.C., Yoon, I.K., Warren, R.L. and Cross, A.S. (1994). Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cyclic AMP. *Infect. Immun.* **62,** 4432–4439.

Inglesby, T.V. *et al.* (2002). Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA* **287,** 2236–2252.

Ivins, B.E., Ezzell, J.W., Jr., Jemski, J., Hedlund, K.W., Ristroph, J.D. and Leppla, S.H. (1986). Immunization studies with attenuated strains of *Bacillus anthracis*. *Infect. Immun.* **52,** 454–458.

Ivins, B.E. and Welkos, S.L. (1986). Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. *Infect. Immun.* **54,** 537–542.

Ivins, B.E. and Welkos, S.L. (1988). Recent advances in the development of an improved, human anthrax vaccine. *Eur. J. Epidemiol.* **4,** 12–19.

Keppie, J., Harris-Smith, P.W. and Smith, H. (1963). The chemical basis of the virulence of *Bacillus anthracis*. IX. Its aggressins and their mode of action. *Brit. J. Exp. Path.* **44,** 446–453.

Kim, S.O., Jing, Q., Hoebe, K., Beutler, B., Duesbery, N.S. and Han, J. (2002). Sensitizing anthrax lethal toxin-resistant macrophages to lethal toxin-induced killing by tumor necrosis factor-alpha. *J. Biol. Chem.* **278,** 7413–7421.

Kimura, K., Kubota, T., Ohishi, I., Isogai, E., Isogai, H. and Fujii, N. (1998). The gene for component-II of botulinum C2 toxin. *Vet. Microbiol.* **62,** 27–34.

Kirby, J.E. (2004). Anthrax lethal toxin induces human endothelial cell apoptosis. *Infect. Immun.* **72,** 430–439.

Klimpel, K.R., Arora, N. and Leppla, S.H. (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence, which is required for lethal toxin activity. *Mol. Microbiol.* **13,** 1093–1100.

Klimpel, K.R., Molloy, S.S., Thomas, G. and Leppla, S.H. (1992). Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10277–10281.

Koehler, T.M. (2002a). *Bacillus anthracis* genetics and virulence gene regulation. *Curr. Top. Microbiol. Immunol.* **271,** 143–164.

Koehler, T.M. (2002b). *Anthrax*. Springer, Berlin.

Koehler, T.M., Dai, Z. and Kaufman-Yarbray, M. (1994). Regulation of the *Bacillus anthracis* protective antigen gene: CO2 and a transacting element activate transcription from one of two promoters. *J. Bacteriol.* **176,** 586–595.

Koo, H.M., VanBrocklin, M., McWilliams, M.J., Leppla, S.H., Duesbery, N.S. and Woude, G.F. (2002). Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase kinase. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 3052–3057.

Kumar, P., Ahuja, N. and Bhatnagar, R. (2002). Anthrax edema toxin requires influx of calcium for inducing cyclic AMP toxicity in target cells. *Infect. Immun.* **70,** 4997–5007.

Kumar, P., Ahuja, N. and Bhatnagar, R. (2001). Purification of anthrax edema factor from *Escherichia coli* and identification of residues required for binding to anthrax protective antigen. *Infect. Immun.* **69,** 6532–6536.

Labruyere, E., Mock, M., Ladant, D., Michelson, S., Gilles, A.M., Laoide, B. and Barzu, O. (1990). Characterization of ATP and calmodulin-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase. *Biochemistry* **29,** 4922–4928.

Labruyere, E., Mock, M., Surewicz, W.K., Mantsch, H.H., Rose, T., Munier, H., Sarfati, R.S. and Barzu, O. (1991). Structural and ligand-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase and of a catalytically inactive variant in which glutamine substitutes for lysine-346. *Biochemistry* **30,** 2619–2624.

Lacy, D.B., Mourez, M., Fouassier, A. and Collier, R.J. (2002). Mapping the anthrax protective antigen binding site on the lethal and edema factors. *J. Biol. Chem.* **277,** 3006–3010.

Lacy, D.B., Wigelsworth, D.J., Melnyk, R.A., Harrison, S.C. and Collier, R.J. (2004a). Structure of heptameric protective antigen bound to an anthrax toxin receptor: A role for receptor in pH-dependent pore formation. *Proc. Natl Acad. Sci. U. S. A.* **101,** 13147–13151.

Lacy, D.B., Wigelsworth, D.J., Scobie, H.M., Young, J.A. and Collier, R.J. (2004b). Crystal structure of the von Willebrand factor A domain of human capillary morphogenesis protein 2: An anthrax toxin receptor. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6367–6372.

Laird, M.W. *et al.* (2004). Production and purification of *Bacillus anthracis* protective antigen from *Escherichia coli*. *Protein Expr. Purif.* **38,** 145–152.

Lane, H.C., Montagne, J.L. and Fauci, A.S. (2001). Bioterrorism: A clear and present danger. *Nat. Med.* **7,** 1271–1273.

Leppla, S.H. (2000). Anthrax toxin. In: *Bacterial Protein Toxins* (eds. K. Aktories and I. Just) pp. 445–472. Springer, Berlin.

Leppla, S.H. (1988). Production and purification of anthrax toxin. In: *Methods in Enzymology, Vol. 165* (ed. S. Harshman) pp. 103–116. Academic Press, Inc., Orlando, FL.

Leppla, S.H. (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. USA.* **79,** 3162–3166.

Leppla, S.H. (1984). *Bacillus anthracis* calmodulin-dependent adenylate cyclase: chemical and enzymatic properties and interactions with eucaryotic cells. In: *Advances in Cyclic Nucleotide and Protein Phosphorylation Research, Vol 17* (ed. P. Greengard) pp. 189–198. Raven Press, New York.

Leppla, S.H. (1991a). Purification and characterization of adenylyl cyclase from *Bacillus anthracis*. In: *Methods in Enzymology, Vol 195* (eds. R. A. Johnson and J. D. Corbin) pp. 153–168. Academic Press, San Diego, CA.

Leppla, S.H. (1991b). The anthrax toxin complex. In: *Sourcebook of Bacterial Protein Toxins* (eds. J. E. Alouf and J. H. Freer) pp. 277–302. Academic Press, London.

Leppla, S.H., Friedlander, A.M., and Cora, E. (1988). Proteolytic activation of anthrax toxin bound to cellular receptors. In: *Bacterial Protein Toxins* (eds. F. Fehrenbach, J. E. Alouf, P. Falmagne, W. Goebel, J. Jeljaszewicz, D. Jurgen, and R. Rappuoli) pp. 111–112. Gustav Fischer, New York.

Little, S.F. and Ivins, B.E. (1999). Molecular pathogenesis of *Bacillus anthracis* infection. *Microbes. Infect.* **1,** 131–139.

Little, S.F., Ivins, B.E., Fellows, P.F. and Friedlander, A.M. (1997). Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. *Infect. Immun.* **65,** 5171–5175.

Little, S.F., Leppla, S.H., Burnett, J.W. and Friedlander, A.M. (1994). Structure-function analysis of *Bacillus anthracis* edema factor by using monoclonal antibodies. *Biochem. Biophys. Res. Commun.* **199,** 676–682.

Little, S.F., Leppla, S.H. and Cora, E. (1988). Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. *Infect. Immun.* **56,** 1807–1813.

Little, S.F., Leppla, S.H. and Friedlander, A.M. (1990). Production and characterization of monoclonal antibodies against the lethal factor component of *Bacillus anthracis* lethal toxin. *Infect. Immun.* **58,** 1606–1613.

Little, S.F., Novak, J.M., Lowe, J.R., Leppla, S.H., Singh, Y., Klimpel, K.R., Lidgerding, B.C. and Friedlander, A.M. (1996). Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Microbiol.* **142,** 707–715.

Liu, S., Aaronson, H., Mitola, D.J., Leppla, S.H. and Bugge, T.H. (2003). Potent antitumor activity of a urokinase-activated engineered anthrax toxin. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 657–662.

Liu, S., Bugge, T.H. and Leppla, S.H. (2001). Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. *J. Biol. Chem.* **276,** 17976–17984.

Liu, S. and Leppla, S.H. (2002). Cell surface tumor endothelium marker 8 cytoplasmic tail-independent anthrax toxin binding, proteolytic processing, oligomer formation, and internalization. *J. Biol. Chem.* **278,** 5227–5234.

Lu, Y., Friedman, R., Kushner, N., Doling, A., Thomas, L., Touzjian, N., Starnbach, M. and Lieberman, J. (2000). Genetically modified anthrax lethal toxin safely delivers whole HIV protein antigens into the cytosol to induce T cell immunity. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8027–8032.

Makino, S., Watarai, M., Cheun, H.I., Shirahata, T. and Uchida, I. (2002). Effect of the lower molecular capsule released from the cell surface of *Bacillus anthracis* on the pathogenesis of anthrax. *J. Infect. Dis.* **186,** 227–233.

McAllister, R.D., Singh, Y., Du Bois, W.D., Potter, M., Boehm, T., Meeker, N.D., Fillmore, P.D., Anderson, L.M., Poynter, M.E. and Teuscher, C. (2003). Susceptibility to anthrax lethal toxin is controlled by three linked quantitative trait Loci. *Am. J. Pathol.* **163,** 1735–1741.

Menard, A., Altendorf, K., Breves, D., Mock, M. and Montecucco, C. (1996a). The vacuolar ATPase proton pump is required for the cytotoxicity of *Bacillus anthracis* lethal toxin. *FEBS Lett.* **386,** 161–164.

Menard, A., Papini, E., Mock, M. and Montecucco, C. (1996b). The cytotoxic activity of *Bacillus anthracis* lethal factor is inhibited by leukotriene A4 hydrolase and metallopeptidase inhibitors. *Biochem. J.* **320,** 687–691.

Meynell, E. and Meynell, G.G. (1964). The roles of serum and carbon dioxide in capsule formation by *Bacillus anthracis*. *J. Gen. Microbiol.* **34,** 153–164.

Mignot, T., Couture-Tosi, E., Mesnage, S., Mock, M. and Fouet, A. (2004). In vivo *Bacillus anthracis* gene expression requires PagR as an intermediate effector of the AtxA signalling cascade. *Int. J. Med. Microbiol.* **293,** 619–624.

Mikesell, P., Ivins, B.E., Ristroph, J.D. and Dreier, T.M. (1983). Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.* **39,** 371–376.

Miller, C.J., Elliott, J.L. and Collier, R.J. (1999). Anthrax protective antigen: prepore-to-pore conversion. *Biochemistry* **38,** 10432–10441.

Miller, J., McBride, B.W., Manchee, R.J., Moore, P. and Baillie, L.W.J. (1998). Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. *Lett. Appl. Microbiol.* **26,** 56–60.

Milne, J.C., Blanke, S.R., Hanna, P.C. and Collier, R.J. (1995). Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol.* **15,** 661–666.

Milne, J.C. and Collier, R.J. (1993). pH-dependent permeabilization of the plasma membrane of mammalian cells by anthrax protective antigen. *Mol. Microbiol.* **10,** 647–653.

Milne, J.C., Furlong, D., Hanna, P.C., Wall, J.S. and Collier, R.J. (1994). Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J. Biol. Chem.* **269,** 20607–20612.

Moayeri, M., Haines, D., Young, H.A. and Leppla, S.H. (2003). *Bacillus anthracis* lethal toxin induces TNF-{alpha}-independent hypoxia-mediated toxicity in mice. *J. Clin. Invest.* **112,** 670–682.

Moayeri, M. and Leppla, S.H. (2004). The roles of anthrax toxin in pathogenesis. *Curr. Opin. Microbiol.* **7,** 19–24.

Moayeri, M., Martinez, N.W., Wiggins, J., Young, H.A. and Leppla, S.H. (2004). Mouse susceptibility to anthrax lethal toxin is influenced by genetic factors in addition to those controlling macrophage sensitivity. *Infect. Immun.* **72,** 4439–4447.

Mock, M., Labruyere, E., Glaser, P., Danchin, A. and Ullmann, A. (1988). Cloning and expression of the calmodulin-sensitive *Bacillus anthracis* adenylate cyclase in *Escherichia coli*. *Gene* **64,** 277–284.

Mock, M. and Mignot, T. (2003). Anthrax toxins and the host: a story of intimacy. *Cell Microbiol.* **5,** 15–23.

Mogridge, J., Cunningham, K. and Collier, R.J. (2002a). Stoichiometry of anthrax toxin complexes. *Biochemistry* **41,** 1079–1082.

Mogridge, J., Cunningham, K., Lacy, D.B., Mourez, M. and Collier, R.J. (2002b). The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7045–7048.

Mogridge, J., Mourez, M. and Collier, R.J. (2001). Involvement of domain 3 in oligomerization by the protective antigen moiety of anthrax toxin. *J. Bacteriol.* **183,** 2111–2116.

Mourez, M. (2004). Anthrax toxins. *Rev. Physiol Biochem Pharmacol.* **152,** 135–164.

Mourez, M. *et al.* (2003). Mapping dominant-negative mutations of anthrax protective antigen by scanning mutagenesis. *Proc. Natl Acad. Sci. U. S. A.* **100,** 13803–13808.

Nanda, A. and St Croix, B. (2004). Tumor endothelial markers: new targets for cancer therapy. *Curr. Opin. Oncol.* **16,** 44–49.

Nassi, S., Collier, R.J. and Finkelstein, A. (2002). PA63 channel of anthrax toxin: An extended beta-barrel. *Biochemistry* **41,** 1445–1450.

Novak, J.M., Stein, M.P., Little, S.F., Leppla, S.H. and Friedlander, A.M. (1992). Functional characterization of protease-treated *Bacillus anthracis* protective antigen. *J. Biol. Chem.* **267,** 17186–17193.

O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J. and Leppla, S. (1985). Effects of anthrax toxin components on human neutrophils. *Infect. Immun.* **47,** 306–310.

Okinaka, R.T. *et al.* (1999). Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. *J. Bacteriol.* **181,** 6509–6515.

Panchal, R.G. *et al.* (2004). Identification of small molecule inhibitors of anthrax lethal factor. *Nat. Struct. Mol. Biol.* **11,** 67–72.

Pannifer, A.D. *et al.* (2001). Crystal structure of the anthrax lethal factor. *Nature* **414,** 229–233.

Pannucci, J., Okinaka, R.T., Williams, E., Sabin, R., Ticknor, L.O. and Kuske, C.R. (2002). DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria. *BMC. Genomics* **3,** 34.

Park, J.M., Greten, F.R., Li, Z.W. and Karin, M. (2002). Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase Inhibition. *Science* **297,** 2048–2051.

Park, S. and Leppla, S.H. (2000). Optimized production and purification of *Bacillus anthracis* lethal factor. *Protein Expr. Purif.* **18,** 293–302.

Parkhill, J. *et al.* (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413,** 523–527.

Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M. and Montecucco, C. (2000). Lethal factor of *Bacillus anthracis* cleaves the N-terminus of MAPKKs: analysis of the intracellular consequences in macrophages. *Int. J. Med. Microbiol.* **290,** 421–427.

Perelle, S., Gibert, M., Boquet, P. and Popoff, M.R. (1995). Characterization of *Clostridium perfringens* Iota-toxin genes and expression in *Escherichia coli*. *Infect. Immun.* **63,** 4967.

Perelle, S., Gibert, M., Bourlioux, P., Corthier, G. and Popoff, M.R. (1997). Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. *Infect. Immun.* **65,** 1402–1407.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Pezard, C., Berche, P. and Mock, M. (1991). Contribution of individual toxin components to virulence of *Bacillus anthracis*. *Infect. Immun.* **59,** 3472–3477.

Pezard, C., Duflot, E. and Mock, M. (1993). Construction of *Bacillus anthracis* mutant strains producing a single toxin component. *J. Gen. Microbiol.* **139,** *( Pt 10)*, 2459–2463.

Pollack, M.R. and Richmond, M.H. (1962). Low cysteine content of bacterial extracellular proteins: its possible physiological significance. *Nature* **194,** 446–449.

Popoff, M.R. and Boquet, P. (1988). *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. *Biochem. Biophy. Res. Comm.* **152,** 1361–1368.

Price, L.B., Hugh-Jones, M., Jackson, P.J. and Keim, P. (1999). Genetic diversity in the protective antigen gene of *Bacillus anthracis*. *J. Bacteriol.* **181,** 2358–2362.

Puziss, M., Manning, L.C., Lynch, J.W., Barclay, E., Abelow, I. and Wright, G.G. (1963). Large-scale production of protective antigen of *Bacillus anthracis* in anaerobic cultures. *Appl. Microbiol.* **11,** 330–334.

Quinn, C.P., Shone, C.C., Turnbull, P.C. and Melling, J. (1988). Purification of anthrax-toxin components by high-performance anion-exchange, gel-filtration, and hydrophobic-interaction chromatography. *Biochem. J.* **252,** 753–758.

Quinn, C.P., Singh, Y., Klimpel, K.R. and Leppla, S.H. (1991). Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. *J. Biol. Chem.* **266,** 20124–20130.

Ramirez, D.M., Leppla, S.H., Schneerson, R. and Shiloach, J. (2002). Production, recovery, and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*. *J. Ind. Microbiol. Biotechnol.* **28,** 232–238.

Ratts, R., Zeng, H., Berg, E.A., Blue, C., McComb, M.E., Costello, C.E., vanderSpek, J.C. and Murphy, J.R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J. Cell Biol.* **160,** 1139–1150.

Read, T.D. *et al.* (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423,** 81–86.

Ren, G., Quispe, J., Leppla, S.H. and Mitra, A.K. (2004). Large-scale structural changes accompany binding of lethal factor to anthrax protective antigen; a cryo-electron microscopic study. *Structure* (Camb.) **12,** 2059–2066.

Ristroph, J.D. and Ivins, B.E. (1983). Elaboration of *Bacillus anthracis* antigens in a new, defined culture medium. *Infect. Immun.* **39,** 483–486.

Robertson, D.L. and Leppla, S.H. (1986). Molecular cloning and expression in *Escherichia coli* of the lethal factor gene of *Bacillus anthracis*. *Gene* **44,** 71–78.

Robertson, D.L., Tippetts, M.T. and Leppla, S.H. (1988). Nucleotide sequence of the *Bacillus anthracis* edema factor gene (*cya*): a calmodulin-dependent adenylate cyclase. *Gene* **73,** 363–371.

Rosovitz, M.J., Schuck, P., Varughese, M., Chopra, A.P., Mehra, V., Singh, Y., McGinnis, L.M. and Leppla, S.H. (2003). Alanine scanning mutations in domain 4 of anthrax toxin protective antigen reveal residues important for binding to the cellular receptor and to a neutralizing monoclonal antibody. *J. Biol. Chem.* **278,** 30936–30944.

Santelli, E., Bankston, L.A., Leppla, S.H. and Liddington, R.C. (2004). Crystal structure of a complex between anthrax toxin and its host cell receptor. *Nature* **430,** 905–908.

Sawada-Hirai, R., Jiang, I., Wang, F., Sun, S., Nedellec, R., Ruther, P., Alvarez, A., Millis, D., Morrow, P.R. and Kang, A.S. (2004). Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed. *J. Immune. Based. Ther. Vaccines.* **2,** 5.

Scobie, H.M., Rainey, G.J., Bradley, K.A. and Young, J.A. (2003). Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 5170–5174.

Sellman, B.R., Mourez, M. and Collier, R.J. (2001). Dominant-negative mutants of a toxin subunit: an approach to therapy of anthrax. *Science* **292,** 695–697.

Sharma, M., Swain, P.K., Chopra, A.P., Chaudhary, V.K. and Singh, Y. (1996). Expression and purification of anthrax toxin protective antigen from *Escherichia coli. Protein Expr. Purif.* **7,** 33–38.

Shen, Y., Zhukovskaya, N.L., Guo, Q., Florian, J. and Tang, W.J. (2005). Calcium-independent calmodulin binding and two-metal-ion catalytic mechanism of anthrax edema factor. *EMBO J.* 24, 929–941.

Shen, Y., Lee, Y.S., Soelaiman, S., Bergson, P., Lu, D., Chen, A., Beckingham, K., Grabarek, Z., Mrksich, M. and Tang, W.J. (2002). Physiological calcium concentrations regulate calmodulin binding and catalysis of adenylyl cyclase exotoxins. *EMBO J.* **21,** 6721–6732.

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J. and Pang, Y. (2004). Expression of vip1/vip2 genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. *J. Appl. Microbiol*. **97,** 757–765.

Singh, Y., Chaudhary, V.K. and Leppla, S.H. (1989). A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo. J. Biol. Chem*. **264,** 19103–19107.

Singh, Y., Khanna, H., Chopra, A.P. and Mehra, V. (2001). A dominant negative mutant of *Bacillus anthracis* protective antigen inhibits anthrax toxin action *in vivo. J. Biol. Chem*. **276,** 22090–22094.

Singh, Y., Klimpel, K.R., Arora, N., Sharma, M. and Leppla, S.H. (1994). The chymotrypsin-sensitive site, FFD315, in anthrax toxin protective antigen is required for translocation of lethal factor. *J. Biol. Chem*. **269,** 29039–29046.

Singh, Y., Klimpel, K.R., Goel, S., Swain, P.K. and Leppla, S.H. (1999). Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. *Infect. Immun*. **67,** 1853–1859.

Singh, Y., Klimpel, K.R., Quinn, C.P., Chaudhary, V.K. and Leppla, S.H. (1991). The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. *J. Biol. Chem*. **266,** 15493–15497.

Singh, Y., Leppla, S.H., Bhatnagar, R. and Friedlander, A.M. (1990). Basis of cellular sensitivity and resistance to anthrax lethal toxin. *Salisbury Med. Bull*. **68,** 46–48.

Smith, H., Keppie, J. and Stanley, J.L. (1955). The chemical basis of the virulence of *Bacillus anthracis*. V. the specific toxin produced by *B. anthracis in vivo. Brit. J. Exp. Path*. **36,** 460–472.

Smith, H. and Stoner, H.B. (1967). Anthrax toxic complex. *Fed. Proc*. **26,** 1554–1557.

Soelaiman, S., Wei, B.Q., Bergson, P., Lee, Y.S., Shen, Y., Mrksich, M., Shoichet, B.K. and Tang, W.J. (2003). Structure-based inhibitor discovery against adenylyl cyclase toxins from pathogenic bacteria that cause anthrax and whooping cough. *J. Biol. Chem*. **278,** 25990–25997.

St Croix, B. *et al*. (2000). Genes expressed in human tumor endothelium. *Science* **289,** 1197–1202.

Sterne, M. (1937). Variation in *Bacillus anthracis. Onderstepoort J. Vet. Sci. Anim. Ind*. **8,** 271–349.

Strange, R.E. and Thorne, C.B. (1958). Further purification of the protective antigen of *Bacillus anthracis* produced *in vitro. J. Bacteriol*. **76,** 192–201.

Tasken, K. and Aandahl, E.M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol. Rev*. **84,** 137–167.

Thorne, C.B. (1985). Genetics of *Bacillus anthracis*. In: *Microbiology—85*, (eds. L. Lieve, P. F. Bonventre, J. A. Morello, S. Schlessinger, S. D. Silver, and H. C. Wu) pp. 56–62. American Society for Microbiology, Washington, D.C.

Thorne, C.B. (1993). *Bacillus anthracis*. In: *Bacillus subtilis and other Gram-Positive Bacteria: biochemistry, physiology, and molecular genetics* (eds. A. B. Sonenshein, J. A. Hoch, and R. Losick) pp. 113–124. American Society for Microbiology, Washington, D.C.

Tinsley, E., Naqvi, A., Bourgogne, A., Koehler, T.M. and Khan, S.A. (2004). Isolation of a minireplicon of the virulence plasmid pXO2 of *Bacillus anthracis* and characterization of the plasmid-encoded RepS replication protein. *J. Bacteriol*. **186,** 2717–2723.

Tippetts, M.T. and Robertson, D.L. (1988). Molecular cloning and expression of the *Bacillus anthracis* edema factor toxin gene: a calmodulin-dependent adenylate cyclase. *J. Bacteriol*. **170,** 2263–2266.

Tonello, F., Seveso, M., Marin, O., Mock, M. and Montecucco, C. (2002). Pharmacology: Screening inhibitors of anthrax lethal factor. *Nature* **418,** 386.

Turk, B.E., Wong, T.Y., Schwarzenbacher, R., Jarrell, E.T., Leppla, S.H., Collier, R.J., Liddington, R.C. and Cantley, L.C. (2004). The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. *Nat. Struct. Mol. Biol*. **11,** 60–66.

Turnbull, P.C. (2002). Introduction: anthrax history, disease, and ecology. *Curr. Top. Microbiol. Immunol*. **271,** 1–19.

Turnbull, P.C.B. (1996). Proceedings of the International Workshop on Anthrax, Winchester, England, Sept 19–21, 1995. *Salisbury Med. Bull*. **87,** *Spec. Suppl*., 1–139.

Turnbull, P.C.B. (1990). Terminal bacterial and toxin levels in the blood of guinea pigs dying of anthrax. *Salisbury Med. Bull*. **68,** 53–55.

Uchida, I., Hashimoto, K. and Terakado, N. (1986). Virulence and immunogenicity in experimental animals of *Bacillus anthracis* strains harboring or lacking 110 MDa and 60 MDa plasmids. *J. Gen. Microbiol*. **132,** 557–559.

Uchida, I., Hornung, J.M., Thorne, C.B., Klimpel, K.R. and Leppla, S.H. (1993). Cloning and characterization of a gene whose product is a transactivator of anthrax toxin synthesis. *J. Bacteriol*. **175,** 5329–5338.

Uchida, I., Sekizaki, T., Hashimoto, K. and Terakado, N. (1985). Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. *J. Gen. Microbiol*. **131,** 363–367.

Ulmer, T.S., Soelaiman, S., Li, S., Klee, C.B., Tang, W.J. and Bax, A. (2003). Calcium dependence of the interaction between calmodulin and anthrax edema factor. *J. Biol. Chem*. **278,** 29261–29266.

Varughese, M., Teixeira, A.V., Liu, S. and Leppla, S.H. (1999). Identification of a receptor-binding region within domain 4 of the protective antigen component of anthrax toxin. *Infect. Immun*. **67,** 1860–1865.

Vitale, G., Bernardi, L., Napolitani, G., Mock, M. and Montecucco, C. (2000). Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. *Biochem. J*. **352** *Pt 3*, 739–745.

Vitale, G., Pellizzari, R., Recchi, C., Napolitani, G., Mock, M. and Montecucco, C. (1998). Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophy. Res. Comm*. **248,** 706–711.

Vodkin, M.H. and Leppla, S.H. (1983). Cloning of the protective antigen gene of *Bacillus anthracis. Cell* **34,** 693–697.

Wade, B.H., Wright, G.G., Hewlett, E.L., Leppla, S.H. and Mandell, G.L. (1985). Anthrax toxin components stimulate chemotaxis of

human polymorphonuclear neutrophils. *Proc. Soc. Exp. Biol. Med*. **179,** 159–162.

Watters, J.W., Dewar, K., Lehoczky, J., Boyartchuk, V. and Dietrich, W.F. (2001). Kif1C, a kinesin-like motor protein, mediates mouse macrophage resistance to anthrax lethal factor. *Curr. Biol*. **11,** 1503–1511.

Webster, J.I., Tonelli, L.H., Moayeri, M., Simons, S.S., Jr., Leppla, S.H. and Sternberg, E.M. (2003). Anthrax lethal factor represses glucocorticoid and progesterone receptor activity. *Proc. Natl. Acad. Sci. U. S. A*. **100,** 5706–5711.

Weinstein, J.N. *et al*. (1997). An information-intensive approach to the molecular pharmacology of cancer. *Science* **275,** 343–349.

Welkos, S.L. and Friedlander, A.M. (1988). Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. *Microb. Pathog*. **5,** 127–139.

Welkos, S.L., Lowe, J.R., Eden-McCutchan, F., Vodkin, M., Leppla, S.H. and Schmidt, J.J. (1988). Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis. Gene* **69,** 287–300.

Welkos, S.L., Vietri, N.J. and Gibbs, P.H. (1993). Non-toxigenic derivatives of the Ames strain of *Bacillus anthracis* are fully virulent for mice: role of plasmid pX02 and chromosome in strain-dependent virulence. *Microb. Pathog*. **14,** 381–388.

Wesche, J., Elliott, J.L., Falnes, P.O., Olsnes, S. and Collier, R.J. (1998). Characterization of membrane translocation by anthrax protective antigen. *Biochemistry* **37,** 15737–15746.

Wigelsworth, D.J., Krantz, B.A., Christensen, K.A., Lacy, D.B., Juris, S.J. and Collier, R.J. (2004). Binding stoichiometry and kinetics of the interaction of a human anthrax toxin receptor, CMG2, with protective antigen. *J. Biol. Chem*. **279,** 23349–23356.

Wild, M.A., Xin, H., Maruyama, T., Nolan, M.J., Calveley, P.M., Malone, J.D., Wallace, M.R. and Bowdish, K.S. (2003). Human antibodies from immunized donors are protective against anthrax toxin *in vivo. Nat. Biotechnol*. **21,** 1305–1306.

Wright, G.G., Read, P.W. and Mandell, G.L. (1988). Lipopolysaccharide releases a priming substance from platelets that augments the oxidative response of polymorphonuclear neutrophils to chemotactic peptide. *J. Infect. Dis*. **157,** 690–696.

Yahr, T.L., Vallis, A.J., Hancock, M.K., Barbieri, J.T. and Frank, D.W. (1998). ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc. Natl. Acad. Sci. U. S. A*. **95,** 13899–13904.

Zhang, S., Finkelstein, A. and Collier, R.J. (2004a). Evidence that translocation of anthrax toxin's lethal factor is initiated by entry of its N terminus into the protective antigen channel. *Proc Natl Acad Sci U. S. A*. **101,** 16756–16761.Zhang, S., Udho, E., Wu, Z., Collier, R.J. and Finkelstein, A. (2004b). Protein translocation through anthrax toxin channels formed in planar lipid bilayers. *Biophys. J*. **87,** 3842–3849.

CHAPTER

# 19

# Attack of the nervous system by clostridial toxins: physical findings, cellular and molecular actions

*Bernard Poulain, Bradley G. Stiles, Michel R. Popoff, and Jordi Molgo*

## INTRODUCTION

This chapter summarizes the known pathophysiological and molecular actions on nerve terminals and nerve tissue of several potent toxins produced by *Clostridium species*. This includes the botulinum and tetanus neurotoxins, which are to date the best documented toxins acting on neurotransmitter release. These metalloproteases specifically target neurons (see Chapter 15, this volume), where they cleave the SNARE proteins involved in neuroexocytosis. Although their molecular mechanisms of action were deciphered a decade ago, progress in understanding the exocytotic mechanisms makes an update of the molecular, cellular, and tissual consequences of their actions necessary. During the last decade, many proteins have been identified as playing a role in exocytosis. This is the case for small GTPases of the Rho and Ras subfamilies and, not surprisingly, the several bacterial toxins that modify these GTPases (see also Chapters 8 and 17, this volume) exert a very potent action on neurotransmission. This has pathophysiological relevance because neurological symptoms are often associated with the pathologies caused by these cytotoxins.

The first part of this chapter is aimed at providing background on neuroexocytotic mechanisms. The two other sections summarize what we know about the above mentioned toxins. To simplify the text, much of the literature data has been summarized in tables. Due to space limitation, we have been obliged to quote only a fraction of the relevant references, and thus given priority to the most recent literature published during the last decade. Many of the valuable earlier papers should have been quoted as well, and their references can be found in the reviews we often invite the reader to consult.

## THE CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN EXOCYTOSIS: AN OVERVIEW

### An overview of neurotransmission

Transmission of information or "orders" along neurons is based on propagated bursts of "action potentials," in which each consists of a depolarized wave in the plasma membrane due to transient opening and closing of voltage-gated, ionic channels. Transfer between neurons, or neurons and target cells (muscle fibers, endocrine cells, etc.) is most often chemical in nature and occurs at highly specialized contact sites termed *synapses*. Here, the release of neurotransmitter molecules by presynatic elements enables activation of receptors localized on the postsynaptic target. Conventional neurotransmitters are acetylcholine (ACh), catecholamines like dopamine or noradrenaline, serotonin, glutamate, γ-aminobutyric acid (GABA), glycine, adenosine-triphosphate (ATP), and numerous peptides such as substance P, calcitonin gene-related

peptide (CGRP), etc. Binding of neurotransmitter molecules to postsynaptic receptors can activate intracellular pathways (metabotropic receptors) or ionic channels (ionotropic receptors). Depending upon the charge of an ion species ($Na^+$, $K^+$, $Ca^{2+}$, $Cl^-$) that flows through the ionic channel/receptor and the transmembrane electrochemical gradient for these ions, activation of a channel/receptor gives rise to inward or outward currents through the plasma membrane. In turn, this causes depolarization or hyperpolarization of the postsynaptic plasma membrane, respectively (i.e., the evoked endplate potential at muscle fibers following stimulation of the motor nerve).

In nerve terminals, transmitter molecules are stored in synaptic vesicles. These small lucent vesicles, approximately 50 nm in diameter and containing small organic molecules, are formed by either budding from the early endosome or recycling of empty vesicles (Jahn and Sudhof, 1999; Jahn *et al.*, 2003; Murthy and De Camilli, 2003). Large dense core vesicles, analogous to the secretory granules present in endo- and exocrine cells, contain a peptide rich matrix and, similar to many cargo vesicles, are formed by budding from the trans-Golgi system. The number of synaptic vesicles per nerve terminal can vary from several tens, to hundreds, of thousands at the motor nerve endings. Synaptic vesicles and certain large dense core vesicles are equipped with a vacuolar-type ATPase that creates a proton gradient that drives specific vesicular transporters, thereby allowing storage of neurotransmitter molecules inside these vesicles. To release their content into the synaptic cleft or lumen of a gland, the synaptic vesicles, large dense core vesicles, or secretory granules must fuse their membranes with the plasmalemma, which then allows content diffusion into the surrounding medium. Neurotransmitter release, as well as hormone secretion, is an exocytotic process triggered by increased cytosolic concentrations of $Ca^{2+}$ ions.

## Mechanisms of exocytosis and SNAREs

More than 100 proteins are implicated in $Ca^{2+}$-dependent exocytosis; however, only a dozen of them participate in the core machinery required for transmitter release, while the others serve regulatory roles (reviewed by Jahn and Sudhof, 1999; Doussau and Augustine, 2000; Rettig and Neher, 2002; Jahn *et al.*, 2003; Murthy and De Camilli, 2003; Bader *et al.*, 2004; Galli and Haucke, 2004). Interestingly, several of these proteins are targeted and disabled by various bacterial toxins. We shall now review how these toxins affect the exocytosis of neurotransmitters. However, in order to address the physiology of poisoning at the molecular level, we summarize the many molecular events involved in this process.

Synaptic vesicles are routed towards the release sites, which in most neurons are identified by a special presynaptic matrix termed as the "active zone." Interaction of synaptic vesicles with the actin filaments, under control of the synapsins, maintains a reservoir of transmitter-loaded vesicles at close proximity to the release sites. This reserve can be mobilized very quickly following phosphorylation of synapsins, thus allowing nerve terminals to support release rates beyond the synaptic vesicle refilling/recycling capacity (Doussau and Augustine, 2000; Garner *et al.*, 2000).

When synaptic vesicles arrive at the fusion site, they bind to the scaffold proteins associated with the "active zone" (Garner *et al.*, 2000). However, when the synaptic vesicles are tethered to the fusion site, they are not yet fusogenic. They must acquire fusion competence, and the needed "priming" corresponds to a sequence of molecular events culminating in assembly of a fusion particle comprised of the soluble *N*-ethylmaleimide-sensitive factor (NSF), attachment protein receptors (SNAREs) found on synaptic vesicles and secretory granules (v-SNAREs: VAMP [vesicle-associated membrane protein]/synaptobrevins) on the target plasma membrane (t-SNAREs: SNAP-25 and syntaxin) (Söllner *et al.*, 1993). In brief, priming is initiated by interaction of the vesicular GTPase Rab3 with the plasma membrane-associated protein Rim (step 1 in Figure 19.1). Rim appears to recruit/activate Munc-13 and, in turn, this triggers de-chaperoning of the t-SNARE syntaxin by Munc-18 (Brose *et al.*, 2000; Garner *et al.*, 2000; Segev, 2001; Gallwitz and Jahn, 2003). Following its de-chaperoning, syntaxin adopts an open configuration that exposes its helical domain H3 and then binds to SNAP-25, the other t-SNARE. SNAP-25 is associated with plasma membrane via palmytoils and has two α-helical arms. Therefore, when SNAP-25 associates with the H3 helix of syntaxin, a heterodimeric complex is formed (step 2 in Figure 19.1). These priming events occur at the plasma membrane level, where the v-SNARE-VAMP adopts an open configuration. Availability for open VAMP is regulated by binding to synaptophysin (an integral vesicle protein), cytosolic $Ca^{2+}$-calmodulin, or other proteins (Quetglas *et al.*, 2000; Pennuto *et al.*, 2003; de Haro *et al.*, 2004). Syntaxin and SNAP-25 constitute an acceptor complex for VAMP, thus generating a four helix bundle (step 3 in Figure 19.1) that is a stable, SDS-resistant, highly packed "rod" of about 10Å diameter (step 4 in Figure 19.1). SNARE complex formation is not enough for promoting fusion (Jahn *et al.*, 2003; Szule and Coorssen, 2003). Indeed, although membranes are pulled into close proximity such that the inner leaflet of the plasma

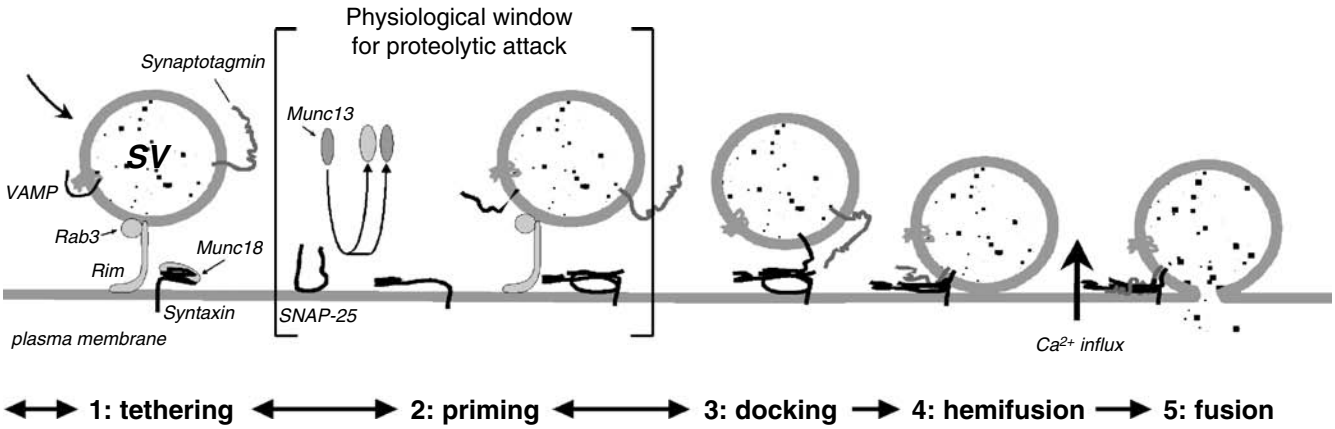



**FIGURE 19.1** Exocytosis is a multiple-step process. Exocytosis follows a sequence of steps during which free synaptic vesicles tether (1) to the plasma membrane via scaffold proteins (not shown). Interaction of Rab3 with Rim signals the onset of priming (2), during which the three SNAREs (VAMP, SNAP-25, and syntaxin) assemble into a complex. Syntaxin is released from Munc-18 and then associates with SNAP-25. Recruitment of VAMP to SNAP-25/syntaxin forms a four helix SNARE complex. Synaptotagmin is also recruited, and at this time synaptic vesicles are "docked" (3). Zippering of the SNARE complex into the highly tight form permits hemifusion (4) of the outer leaflet of synaptic vesicles with the inner leaflet of plasma membranes. $Ca^{2+}$ influx via voltage-gated channels activated by nerve terminal depolarization (i.e. upon action potential arrival) allows $Ca^{2+}$ binding to synaptotagmin and termination of the fusion process (5), which ultimately causes exocytosis of synaptic vesicle content.

membrane mixes with the outer leaflet of the synaptic vesicle membrane, the fusion process seems to stop at this hemifused state.

Termination of fusion is triggered by a rise in intracellular $Ca^{2+}$ levels, which may be caused by $Ca^{2+}$ influx through voltage-gated channels (e.g., at nerve terminals), $Ca^{2+}$ release from intracellular stores, or both (e.g., at many neuroendocrine cells) (Augustine, 2001; Rettig and Neher, 2002; Petersen, 2003). The principal $Ca^{2+}$ sensor for triggering fusion is synaptotagmin, an integral synaptic vesicle protein that interacts in a $Ca^{2+}$-dependent way with SNAREs, notably with the C-terminus of SNAP-25 (Chapman, 2002; Jahn *et al.*, 2003; Koh and Bellen, 2003). Synaptotagmin is equipped with two "C2-domains," which acquire high affinity for membrane lipids upon binding to $Ca^{2+}$ ions. It has been hypothesized that following $Ca^{2+}$ binding to synaptotagmin, the two C2 domains "plunge" into the inner leaflet of the plasma membrane, and this increases local membrane bending. Several SNAREs-synaptotagmin complexes (possibly three) must act in tandem to allow fusion of one synaptic vesicle (Poirier *et al.*, 1998; Hua and Scheller, 2001). Lipids from the outer leaflet of the plasma membrane mix with those from the inner leaflet of the synaptic vesicle membrane, thus enabling fusion pores to then open and release vesicle contents (Jahn *et al.*, 2003), step 5 in Figure 19.1. According to this scenario, formation of the SNARE complex allows pre-fusion of tethered synaptic vesicles, but is not sufficient alone to drive exocytosis. Only the last fusion step is lacking, thus explaining why a delay between the $Ca^{2+}$ rise and vesicle fusion is so brief (< 100μs).

## Actin cytoskeleton and small GTPases in exocytotic mechanisms

Several other proteins play key roles in synaptic vesicle trafficking and priming of tethered synaptic vesicles. Inside nerve terminals, vesicles traffic along actin filaments and this implicates molecular motors like myosins II or V, and small GTPases of the Rab family (Rab3 and others) (Augustine, 2001; Segev, 2001). Reorganization of the actin cytoskeleton is coupled to $Ca^{2+}$-regulated exocytosis in endocrine cells; however, in neurons this is far from being clear (Doussau and Augustine, 2000; Eitzen, 2003; Bader *et al.*, 2004). In secretory cells, but not neurons, the subplasmalemmal actin network serves as a physical barrier, and the transient depolymerization of actin filaments is needed for secretory granule access to fusion sites. Moreover, it is possible that actin activates exocytosis by governing vesicle-granule trafficking towards release sites during fusion events (Eitzen, 2003; Bader *et al.*, 2004).

Organization of the actin-based cytoskeleton is controlled by several proteins, including several small GTPases like the Rho proteins (Hall, 1998) and ADP-ribosylation factor (ARF). Like most small GTPases, ARF and Rho proteins cycle between GDP-bound (inactive) and GTP-bound (active) states, thereby

acting as signal transducers that respond to upstream signals for activating downstream effector molecules to carry out their biological functions.

Rho proteins (Rho, Rac, Cdc42) are widely expressed, monomeric GTPases. Their translocation to specific membrane domains enables intervention of distinct biological functions, which include: (i) regulation of actin cytoskeletal dynamics; (ii) cell cycle progression; (iii) gene transcription; (iv) membrane transport; and (v) exo-/endo-cytosis (Hall, 1998; Bader *et al*., 2004). In chromaffin and PC12 cells, RhoA associates with secretory granules, whereas Rac1 and Cdc42 are found in the subplasmalemmal region (Bader *et al*., 2004). Activation of phosphatidylinositol 4-kinase by RhoA leads to phosphatidylinositol 4,5-bisphosphate (PIP2) residing on the granule membrane. This may promote the formation of granule-associated actin filaments and/or stabilize the subplasmalemmal actin barrier (Bader *et al*., 2004). In chromaffin cells, Cdc42 and Rac1 control actin polymerization and secretion (Li *et al*., 2003; Gasman *et al*., 2004). The Cdc42-controlled pathway seems to specifically target polymerization of local actin filaments at the granule docking sites in order to regulate expansion and/or closure of the fusion pore (Bader *et al*., 2004; Gasman *et al*., 2004). In neurons, Rac1 is associated with synaptic vesicles and plasma membrane (Doussau, 2000). Rac1 is involved in a post-docking step of neuronal exocytosis during which it controls in an all-or-none manner the functionality of release sites (Doussau *et al*., 2000; Humeau *et al*., 2002), possibly via regulation of phospholipase D (PLD) activity (Hammond *et al*., 1997; Humeau *et al*., 2002) (Figure 19.2).

ARF GTPases regulate exocytosis in various cell types including melanotrophs, adipocytes, neutrophils, mast cells, gastric parietal cells, insulin-secreting pancreatic β cells, chromaffin cells, PC12 cells, and neurons (Bader *et al*., 2004). In secretory cells, ARF6 is bound to secretory granules, whereas in neurons, it is on the plasma membrane and associated with recycling of clathrin-coated vesicles (Krauss *et al*., 2003). ARF6 activates plasma membrane PLD (Vitale *et al*., 2002) and PIP5-kinase, leading to the local production of phosphatidic acid (PA) and PIP2. Therefore, ARF6 may act during the priming events by regulating Munc-13 and synaptotagmin, which are also regulated by phosphoinositide levels (Cremona and De Camilli, 2001; Osborne *et al*., 2001).

The Ral GTPase is abundant in nerve terminals and associates with synaptic vesicles (Bielinski *et al*., 1993). This molecule apparently plays a key role in neurotransmitter release by regulating the pool size of readily releasable, synaptic vesicles (Polzin *et al*., 2002). Ral has been implicated in regulating PLD activity (Luo *et al*., 1998).

PLD produces PA and, as mentioned above, is the final target of several pathways implicating small GTPases (ARF6, Ral, and various Rho GTPases). It may be speculated that PLD is activated by these GTPases upon docking of synaptic vesicles, or secretory granules, at the release sites. PLD activation is an important event for exocytosis in neurons and many secretory cell types (Humeau *et al*., 2001a; Vitale *et al*., 2001; Choi *et al*., 2002); and review by Bader *et al*., 2004). PLD-produced PA may activate enzymes and serve as an attachment site for proteins of the fusion machinery, like synaptotagmin or VAMP, or alternatively the PA molecule may act as a precursor for lipid signaling. PA is also a cone-shaped lipid, and biophysical studies have revealed that its local accumulation promotes a negative curvature of the inner (cytoplasmic) plasma membrane leaflet (Chernomordik and Kozlov, 2003). Therefore, it has been hypothesized that PA facilitates formation of the hemifusion intermediates required for vesicle fusion to the plasma membranes, downstream from SNARE complex formation (Humeau *et al*., 2001a; Vitale *et al*., 2001; and review by Chernomordik and Kozlov, 2003) (Figure 19.2).

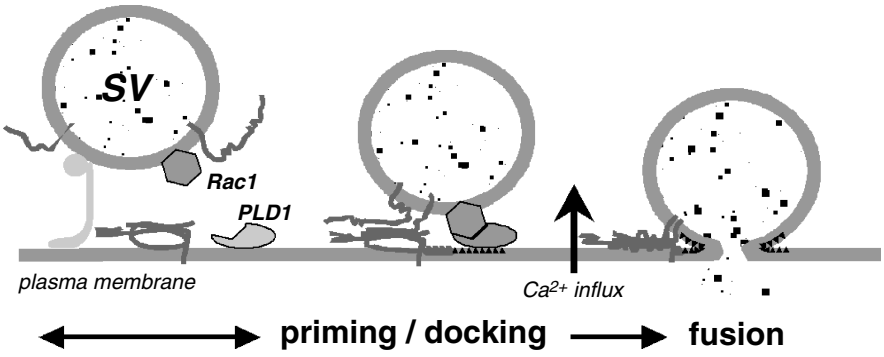



**FIGURE 19.2** Hypothetical model for the role of Rac1 and PLD1 in exocytosis at nerve terminals. Upon synaptic vesicle tethering, vesicular Rac GTPase is activated (i.e., undergoes GDP to GTP exchange) and recruits/activates plasma membrane associated PLD. Local production of cone-shaped phosphatidic acid (denoted here as small, **closed** triangles on the plasma membrane) promotes membrane bending and hemifusion intermediates required for forming exocytotic fusion pores.

## BOTULINUM AND TETANUS NEUROTOXINS

### Toxins from C. *tetani*

Tetanus neurotoxin (TeNT), also called *tetanospasmin*, is produced by *C. tetani* and is responsible for all the neurological disorders of tetanus caused by this bacterium. Only one toxinotype of TeNT is known, and those bacteria producing TeNT display homogeneous bacteriological characteristics that uniformly form *C. tetani*. Some *C. tetani* strains become non-toxigenic and indistinguishable from the toxigenic strains by phenotype and DNA/DNA homology. Toxigenic *C. tetani* strains contain a large plasmid (74 kbp) harboring the *tent* gene.

*C. tetani* also produces a chromosomally encoded hemolysin called tetanolysin O, which belongs to the cholesterol-dependent, pore-forming group of toxins. The complete genome sequence of *C. tetani* has revealed additional virulence factors possibly involved in *C. tetani* invasion into host tissues, which include: (i) two predicted hemolysins; (ii) the ColT collagenase related to *Clostridium histolyticum* collagenases; (iii) a cysteine protease related to the clostripain family; (iv) two fibronectin-binding proteins; (v) three internalin A homologues, which in *Listeria* mediate their entry into host cells; (vi) six surface layer and/or adhesion proteins; and (vii) three membrane proteins related to other bacterial virulence proteins. The ColT molecule is plasmid encoded, while genes for the other putative virulence factors are located on the chromosome (Brüggemann *et al*., 2003).

### Toxins from C. *botulinum* and related *Clostridium species*

#### *Botulinum neurotoxins*

Botulinum neurotoxin (BoNT) was originally defined as a toxin inducing a flaccid, often lethal, paralysis, and the bacteria producing this protein are called *C. botulinum*. However, in contrast to *C. tetani*, the BoNT-producing clostridia are physiologically and genetically heterogeneous and thus belong to several bacteriological groups and species. According to their antigenic properties, BoNTs were identified as seven different toxin types (A, B, C1, D, E, F, and G), and the *C. botulinum* species were divided into four physiological groups:

- group I: *C. botulinum* A, and proteolytic strains of *C. botulinum* B and F.
- group II: *C. botulinum* E, and glucidolytic strains of *C. botulinum* B and F.
- group III: *C. botulinum* C and D.
- group IV: *C. botulinum* G. This group, which also includes non-toxigenic strains previously identified as *Clostridium subterminale* and *Clostridium hastiforme*, is metabolically distinct from the others and has now been assigned to a different species called *Clostridium argentinense* (Suen *et al*., 1988).

*C. botulinum* A, B, and E are mainly involved in human botulism, whereas *C. botulinum* C and D are predominantly responsible for animal botulism (type C in birds and minks, and type D mainly in cattle).

The taxonomic position of *C. botulinum* became even more ambiguous since BoNT is produced by clostridia clearly distinct from *C. botulinum*, yet these strains are biochemically and genetically related to different species such as *Clostridium butyricum* and *Clostridium baratii*. Neurotoxigenic *C. butyricum* strains produce a BoNT/E highly related (96.9% identity at the amino acid level) with those of *C. botulinum* E. These strains have been identified in infants and young people in Italy, as well as during botulism outbreaks throughout China following consumption of fermented paste made of soybeans and wax gourds (Aureli *et al*., 1986; Meng *et al*., 1997; Fenicia *et al*., 1999). Toxigenic *C. baratii* producing a BoNT/F was also responsible for one case of infant, and two cases of adult, botulism in the United States (Hatheway, 1993).

Genes encoding for the different types of BoNT are present on various genetic elements that include phages, plasmids, and chromosomes, depending upon the species and strain. In *C. argentinense*, like in *C. tetani*, the neurotoxin gene is present within a large plasmid. Plasmids of various sizes and bacteriophages are evident in *C. botulinum* A, B, E, and F strains, but toxigenicity has not been associated with these genetic elements. The genes encoding for these neurotoxins are chromosomal but, in neurotoxigenic *C. butyricum* strains, the location of the BoNT/E gene is still undefined. However, in *C. botulinum* C and D, it is clear that BoNT is encoded by bacteriophages. *C. botulinum* C and D strains cured of their phages do not produce BoNT/C1 or BoNT/D, respectively. Such organisms can be converted into neurotoxigenic strains of C or D by respective reinfection with phages obtained from toxigenic *C. botulinum* C or D strains. The BoNT/C1 and BoNT/D genes have been cloned and sequenced from purified phage DNA of *C. botulinum* C-468 and *C. botulinum* D-1873, respectively (Popoff and Marvaud, 1999).

Usually, a *C. botulinum* strain produces only one type of neurotoxin and the locus is present as a single copy on the genome. Rare strains synthesize two types of neurotoxins, such as A-B, B-F, or A-F. In the multiple toxin-producing strains, the neurotoxins are usually

produced at different levels. Thereby, in A-B and B-F producers, 10 times more BoNT/B is synthesized than BoNT/A and BoNT/F. Other strains, although they produce only one BoNT toxinotype, contain an additional silent *bont* gene. Thus, several *C. botulinum* A strains possess a *bont/B* gene containing a proximal stop codon, two amino acid deletions, and two single nucleotide deletions that cause two shifts in the reading frame and multiple stop signals (reviewed in Popoff and Marvaud, 1999).

Overall similarity between the available neurotoxin sequences ranges from 34 to 97% identity. Neurotoxin sequences from the same toxinotype and physiological group are almost identical, except for BoNT/A1 and BoNT/A2, which are highly related (90% identity) yet differ in sequence deletions by 129 residues. BoNTs of the same toxinotype produced by strains from different *C. botulinum* groups or *Clostridium species* possess a high level of identity (70 to 97%). BoNT sequences from *C. botulinum* E and *C. butyricum* share 97% identity. BoNT/B sequences from proteolytic and non-proteolytic *C. botulinum* B share 93% identity, but BoNT/F from *C. botulinum* F and *C. baratii* have only 70% identity. Both variants of BoNT/B seem equipotent in mouse lethal assays. No differences in epidemiology and clinical manifestations have been reported in botulism type B due to proteolytic and non-proteolytic strains. In addition, mosaic structures of *bont* genes have been identified. Molecular characterization of BoNT/D from strain Dsa reveals that the N-terminal region (residues 1 to 522) is highly related to the corresponding region of BoNT/D (96% identity), but the C-terminal region (residues 945 to 1285) is related to the corresponding segment of BoNT/C (74% identity). Inversely, BoNT/C produced by strain C6813 has an N-terminal region related to BoNT/C and a C-terminal region from BoNT/D. However, the central region (residues 523 to 944) remains quite common (83–92% identity) to the hybrid and typical C, as well as D, strains. Thus, genetic analysis suggests that the clostridial BoNT genes are derived from a common ancestor, which has evolved by duplication, mutation, and recombination (reviewed in Popoff and Marvaud, 1999).

### *Additional toxins*

*C. botulinum* C and D strains produce the C2 enterotoxin, which is a binary toxin that ADP-ribosylates monomeric actin within the cytosol and subsequently depolymerizes the actin cytoskeleton. These strains also synthesize an exoenzyme, called C3, which ADP-ribosylates Rho GTPase that leads to disorganization of the actin filaments. C2 toxin genes are located on the chromosome, whereas that of C3 is on phage DNA which also contains the *bont/C1* or *bont/D* genes. To date, the pathological significance of C2 and C3 during *C. botulinum* disease is not clearly known. Finally, various *C. botulinum* strains usually produce a hemolysin, or botunolysin, related to the cholesterol-dependent pore forming toxins (Haque *et al*., 1992) (see Chapter 26 this volume).

## Structure of clostridial neurotoxins

BoNTs and TeNT share a common structure that is first synthesized as a precursor protein (about 150 kDa), which is inactive or weakly active. The precursor, which does not contain signal peptide, is possibly released from the bacterium by a cell-wall exfoliation mechanism. The precursor is proteolytically activated in the extra-bacterial medium either by *Clostridium* proteases or by exogenous proteases, such as those naturally found in the intestinal content. The active neurotoxin consists of a light (L) chain (~ 50 kDa) and a heavy (H) chain (~ 100 kDa), which remain linked by a disulfide bridge. Activation of BoNT/A by an endogeneous protease from *C. botulinum* cleaves between Lys438-Thr439, thus removing 10 amino acids (Thr439-Lys448) (Krieglstein *et al*., 1994). In BoNT/E, cleavage occurs at Lys419-Gly420 and Arg422-Lys423, thus excising a Gly420-Ile-Arg422 peptide (Antharavally and DasGupta, 1997). Proteases produced by *C. tetani* cleave TeNT at Glu449 and Ala456 (Krieglstein *et al*., 1991). For the other BoNTs, cleavage occurs at one or two sites, which include: Arg444/Ser445 and Lys449/Thr450 for BoNT/C; Lys442/Asn443 and Arg445/Asp446 for BoNT/D; as well as Arg435/ Lys436 and Lys439/Ala440 for BoNT/F (Sagane *et al*., 1999).

The crystallographic structures of BoNT/A, BoNT/B, as well as the C-terminal domain of TeNT have been solved and reveal three distinct domains: an L-chain containing α-helices and β-strands that include the catalytic zinc binding motif; the N-terminal part of the H-chain (Hn) forming two unusually long and twisted α-helices; and the C-terminal part of the H-chain (Hc), consisting of two distinct subdomains involved in receptor recognition (Umland *et al*., 1997; Lacy *et al*., 1998; Lacy and Stevens, 1999; Emsley *et al*., 2000; Swaminathan and Eswaramoorthy, 2000; Fotinou *et al*., 2001).

### *The binding domain*

The Hc binding domain of BoNTs and TeNT consists of two distinct subdomains, the N-terminal (Hc-N) and C-terminal (Hc-C) halves, with few protein-protein contacts between them. Hc-N has two seven-stranded β-segments arranged in a jelly roll motif quite similar

to that of lectins. The amino acid sequence of this subdomain is highly conserved among clostridial neurotoxins, thus suggesting a similar folding pattern. The Hc-C subdomain contains a modified β-trefoil folding motif present in several other proteins that possess recognition and binding functions, such as interleukin-1, fibroblast growth factor, and the Kunitz-type trypsin inhibitors. However, the Hc-C sequence is poorly conserved among clostridial neurotoxins. The critical importance of the last 34 residues from Hc-C, and in particular His1293 of TeNT, for binding the oligosaccharide portion of polysialogangliosides was recently demonstrated by photoaffinity labeling (Shapiro *et al*., 1997). In BoNT/B and BoNT/A, one ganglioside binding site was mapped within the Hc-C, whereas TeNT binds simultaneously to two ganglioside molecules (Swaminathan and Eswaramoorthy, 2000; Fotinou *et al*., 2001; Rummel *et al*., 2003; Rummel *et al*., 2004). These data are supportive of a double receptor model of binding for clostridial neurotoxins to the presynaptic membrane. One subdomain binds to a glycoprotein, which differs for the various clostridial neurotoxins, and the other subdomain binds to polysialogangliosides. A major difference between the Hc-C of TeNT and BoNT/A resides in the loop structures, thus suggesting that these external segments may bind to unique protein receptors. However, the Hc-N and Hc-C subdomains are required for neurotoxin interactions with neuronal surface receptors. Thereby, antibodies against Hc-N or Hc-C are poorly protective, whereas antibodies raised against the whole Hc domain efficiently neutralize BoNT/A. The whole BoNT/A Hc domain, and especially the native structure of the interfacial region between Hc-N and Hc-C, is necessary to generate efficiently protective antibodies (Tavallaie *et al*., 2004). In addition, the Hc-C domain of TeNT sufficiently mediates binding to neuronal cells (Herreros *et al*., 2000).

### *The translocation domain*

The Hn regions are highly homologous among the various clostridial neurotoxins, and the conformations of these domains are quite similar in BoNT/A and BoNT/B (Lacy *et al*., 1998; Swaminathan and Eswaramoorthy, 2000). Hn has a cylindrical shape determined by a pair of unusually long (10 nm), twisted α–helices corresponding to peptide segment 685–827 (BoNT/A numbering), which is overall reminiscent of the α-helical hairpin found in some colicins. At both ends of the helical pair, there is a shorter α-helix, which lies parallel to the main helices and, in addition, several strands pack along the two core helices. The segments involved in ion channel formation at low pH remain unidentified, but the overall structure of Hn resembles that of some viral proteins which undergo an acid-driven conformational change (Bullough *et al*., 1994).

### *The catalytic domain*

The metallo-protease domain (L chain, 55 Å × 55 Å × 62 Å) contains both α-helix and β-strand secondary structures, and has little similarity with related enzymes of known structure, apart from the α–helix segment including the zinc binding motif. In addition to the imidazole rings from two histidines of the His-Glu-X-X-d His motif and a water molecule bound to the glutamic acid, the zinc atom is coordinated by another glutamic acid conserved among all clostridial neurotoxins (Rigoni *et al*., 2001; Rossetto *et al*., 2001). The Glu residue is particularly important because it coordinates the water molecule directly implicated in proteolytic hydrolysis. Its mutation leads to complete inactivation of these neurotoxins (Li *et al*., 2000). The active site is similar to that of thermolysin and identifies a primary sphere of residues essential for catalysis, which coincides with the zinc coordinating residues. In addition, there is a secondary layer of amino acids more distant from the zinc center, in which Arg362 and Tyr365 (BoNT/A numbering) are reportedly involved in catalysis by stabilizing the transition state (Rigoni *et al*., 2001; Rossetto *et al*., 2001; Binz *et al*., 2002). The active site of BoNT/A is in a deep recess (20–24 Å) shielded by the H chain; it only becomes accessible to the substrate following reduction of the interchain disulfide bridge, and it appears capable of accommodating a 16 residue segment. Upon reduction at physiological temperature (37°C), BoNT/A loses most of its native tertiary structure and adopts a molten globule conformation that correlates with binding to unstructured SNAP-25 and optimum endopeptidase activity (Cai and Singh, 2001). In contrast, the belt region of BoNT/B formed by the Hc-N domain does not completely occlude the active site, which remains accessible to small molecule inhibitors (Swaminathan and Eswaramoorthy, 2000). However, BoNT/B also requires reduction of the disulfide bridge between L and H chains to activate its endopeptidase activity (Herreros *et al*., 1999; Schiavo *et al*., 2000). Most of the L chain is required for enzymatic activity. The BoNT/A L chain can only tolerate an eight amino acid truncation at the N-terminus, and a 32 amino acid deletion at the C-terminus; however, in contrast the TeNT L chain is still functional following a 16 or even 65 amino acid deletion at the C-terminus (Kurazono *et al*., 1992). The L chain C-terminus likely participates in solubility, stability, and catalysis via stabilizing the tertiary structure (Baldwin *et al*., 2004). Additionally, BoNT/A activity is higher when SNAP-25 is inserted into the membrane

or in liposomes containing negatively charged phospholipids versus soluble SNAP-25, thus indicating that a membrane substrate conformation is better recognized by the toxin and/or phospholipids may facilitate an electrostatic toxin-substrate interaction (Caccin *et al*., 2003).

## Botulinum complexes

BoNTs are associated with non-toxic proteins (ANTPs) to form large complexes also known as progenitor toxins. ANTPs encompass a non-toxic and non-hemagglutinin component (NTNH), as well as several hemagglutinin components (HAs) (reviewed in Oguma *et al*., 1999; Quinn and Minton, 2001; Sharma *et al*., 2003) (Figure 19.3). Botulinum complexes, in which proteins are not covalently linked, are formed in cultures and naturally contaminated food, are stable at acidic pH, and dissociate at alkaline pH (≥pH 8). In contrast, TeNT does not yield any complex, and related botulinum *antp* genes are not evident in the *C. tetani* genome (Brüggemann *et al*., 2003).

*C. botulinum* A produces three types of Botulinum complexes complex designated LL (19S, 900 kDa), L (16S, 500 kDa), and M (12S, 300 kDa). The 12S complex results from a BoNT/A plus NTNH association, and does not possess any hemagglutinating activity. NTNH has a cleavage site within the N-terminus and is

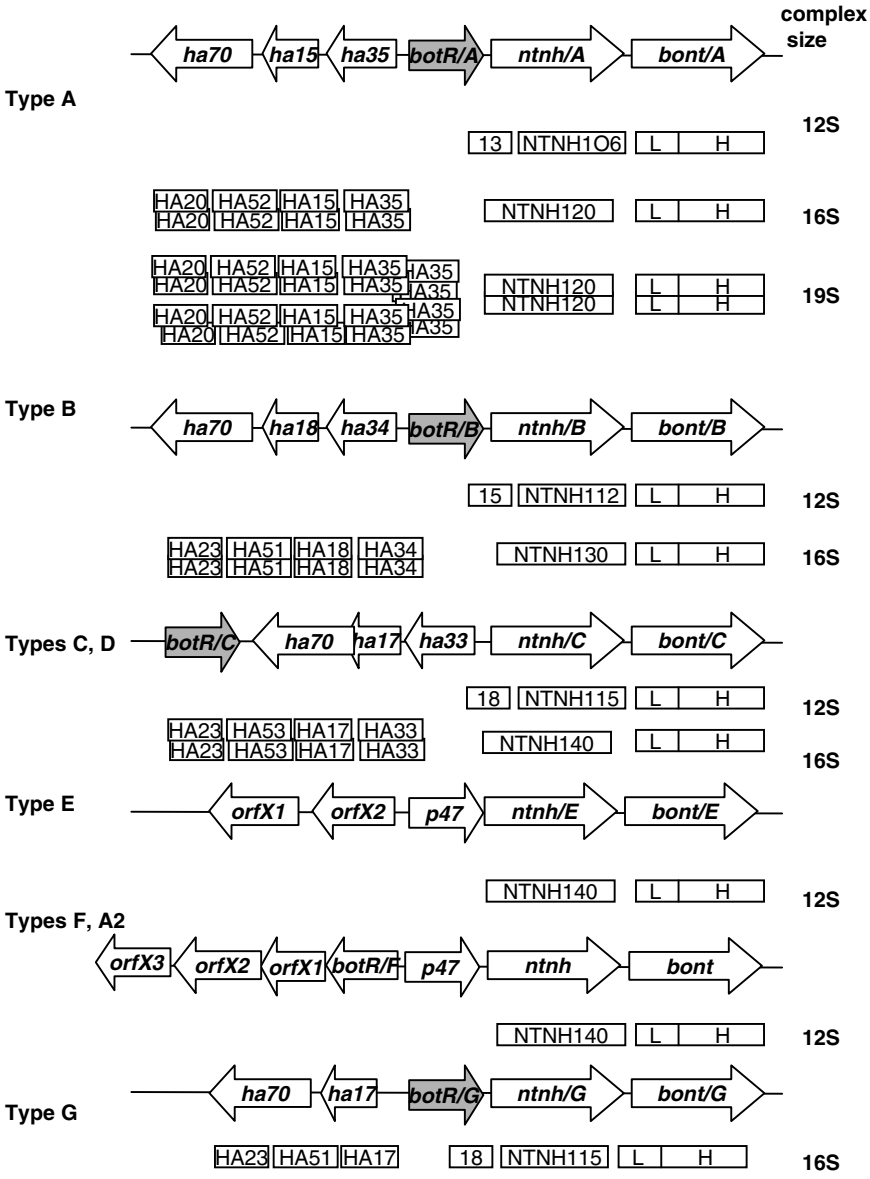



**FIGURE 19.3** Composition of the BoNT complexes from the various *Clostridium botulinum* types.

separated into 13 and 106 kDa fragments as assessed by SDS-PAGE. The 16S complex consists of BoNT/A, NTNH, and HAs (HA33, HA17, and HA70) at a presumed 1:1:2 ratio. HA70 is proteolytically cleaved into HA50 and HA20. The 19S complex has the same protein composition as the 12S complex, but the ratio of HA33 is about twice that found in the 12S complex. The 19S complex is likely a dimer of the 16S complex cross-linked by HA33 (Oguma *et al*., 1999; Sharma *et al*., 2003). The composition of Botulinum complexes types A2 and A1 from strain NCTC2916, which contains different proteins like P47, OrfX1, OrfX2, and OrfX3, remains undetermined (Dineen *et al*., 2003).

*C. botulinum* B, C, and D yield two complex types, L and M, the composition of which is equivalent to those of *C. botulinum* A (Oguma *et al*., 1999; Arimitsu *et al*., 2003). The complete subunit structure of *C. botulinum* D strain 4947 L complex (650 kDa) was determined as one BoNT/D, two HA70, four HA33, and four HA17b molecules, with intermediate complexes (540 and 610 kDa) constituted of various HA subunits. In addition, an intermediate M complex (410 kDa) contains BoNT/D, NTNH, and two molecules of HA70 (Kouguchi *et al*., 2002; Mutoh *et al*., 2003). *C. botulinum* A2, E, and F only produce M complexes devoid of hemagglutinating activity, and *C. argentinense* produces only L complex (Oguma *et al*., 1999).

NTNHs from different *C. botulinum* types possess a high identity level (76–83.5%), and are the most conserved proteins in various Botulinum complexes (Popoff and Marvaud, 1999). NTNH/A, NTNH/C, and NTNH/D contain a cleavage site within their N-terminus, yielding 15 kDa N-terminal and 115 kDa C-terminal fragments. NTNH/A is split into 13 and 106 kDa fragments by cleavage between Pro144/Phe145 (Fujita *et al*., 1995). NTNH/C and NTNH/D are cleaved at Lys127 by a trypsin-like protease with 7–13 amino acids removed from the N-terminus of the 115 kDa fragment that subsequently results in three proteins starting at Leu135, Val139, or Ser141 (Sagane *et al*., 2000). NTNH is only cleaved in the 12S (M) complexes from *C. botulinum* types A, C, and D, but not in the L (16S) or LL (19S) complexes. The cleaved NTNH molecules constitute a nicked structure since the two fragments still remain together after NTNH purification (Sagane *et al*., 2000). In contrast, NTNH/E and NTNH/F show an identical deletion of 33 residues in the corresponding region of NTNH/A, NTNH/C, and NTNH/D encompassing the cutting site, and NTNH/G possesses a slightly different sequence in this region. It is presumed that the processing and additional sequence of NTNH in *C. botulinum* A, C, and D are responsible for forming 12S-, 16S-, and 19S-sized complexes. The inability of *C. botulinum* E and F to form L complexes may result from the absence of HA or other related proteins that bind to NTNH, and from the absence of a putative binding site in NTNH/E and NTNH/F (Oguma *et al*., 1999; Popoff and Marvaud, 1999).

HA33-35 is the most abundant hemagglutinin component of the Botulinum complexes. Type A HA35 binds to oligosaccharides containing galactose-β1-4glucose-*N*-acetyl-D-neuraminic acid (Galβ1-4GlcNAc) (Inoue *et al*., 2001). Thereby, hemagglutination induced by 16S and 19S type A Botulinum complex is mainly mediated through HA35 binding to erythrocyte membrane glycolipids and glycoproteins containing Galβ1-4GlcNAc, such as paragloboside and glycophorin A (Inoue *et al*., 1999; Inoue *et al*., 2001). Similarly, HA33 from types C and D Botulinum complexes bind to paragloboside on Galβ1-4GlcNAc and also sialylglycolipids (GM3), as well as sialoglycoproteins (sialosylparagloboside) on the *N*-acetyl-D-neuraminic acid-α2–3–galactose–β1 motif (Fujinaga *et al*., 2004). The importance of HA33-35 in hemagglutination is also supported by monoclonal antibody studies. Type C-specific monoclonal antibodies against HA33 inhibit hemagglutination, contrary to those against HA50 and HA17 (Mahmut *et al*., 2002b). However, type C HA70 and its derivative HA50 recognize sialosylparagloboside and GM3 at the *N*-acetyl-D-neuraminic acid-α2–3–galactose–β1motif in erythrocyte membranes, like the corresponding 16S Botulinum complex. Thus, HA50 could also be involved in hemagglutination (Fujinaga *et al*., 2004). HA35 purified from *C. botulinum* A is predominantly a dimeric, β-sheet protein in aqueous solutions. In *C. botulinum* A, five N-terminal amino acids are removed from HA35, but similar posttranslational modification has not been observed in HA33 from *C. botulinum C*. The significance of HA35 processing on its biological activity is not known (Sharma *et al*., 1999). It was first discovered that the 31 C-terminal amino acids, which contain a predicted carbohydrate recognition site, play an essential role in hemagglutination (Sagane *et al*., 2001). Structure of type C HA33 shows two β-trefoil domains consisting of a six stranded, antiparallel β-barrel capped on one side by three β-hairpins. Related β-trefoil structures bind to oligosaccharides and are found in other proteins, including various lectins like the ricin B-chain, cytokines, trypsin inhibitor, xylanase, as well as the C-terminal part of BoNTs. Type A HA35 retains a similar structure related to the carbohydrate binding site of ricin, a plant toxin. It is noteworthy that Asp263 and Asn285 of HA35, which are conserved in the lactose-binding site for ricin B chain, are critical for carbohydrate binding (Arndt *et al*., 2005; Inoue *et al*., 2003).

## Tetanus and dissemination of tetanus toxin within the host

### *Tetanus*

Tetanus is a major infectious disease caused by a wound contaminated with *C. tetani*, and it is characterized by persistent tonic muscle spasms with episodes of exacerbation.

Several clinical forms of tetanus have been described: local, generalized, cephalic, and neonatal (Bleck, 1989). Each form has a clinical relevance because of its prognostic value; however, these clinical entities share common cellular and molecular mechanisms. In all these forms, TeNT is the only etiological cause of neurological symptoms. Other secreted proteins like tetanolysin do not act on nerve cells, but their cytotoxicity may favour abscess formation and growth of *C. tetani* in an anaerobic environment.

Although the gut may sometimes offer physiological conditions that promote spore germination of various clostridia, including *C. tetani*, until now there has been no evidence for an intestinal form of tetanus. It has been hypothesized that the lack of associated protective proteins facilitates toxin degradation in the digestive tract. It is also possible that TeNT cannot cross the gut-barrier due to a paucity of receptors that might otherwise enable transepithelial movement (Maksymowych and Simpson, 1998).

### *Dissemination of TeNT and physical findings during tetanus and poisoning experiments*

Local tetanus consists of persistent muscle spasms in close proximity to the infection site. A rare variant is cephalic tetanus, which is caused by *C. tetani* growth in a wound on the head or neck. The spastic paralysis (i.e., a peripheral physical finding) observed during local tetanus has a central origin with disinhibited motoneurons. TeNT cannot cross the blood brain barrier composed of tightly sealed endothelial cells that form the spinal cord and brain capillaries. Compelling evidence (Wellhöner, 1992) has shown that the entry port of TeNT into the central nervous system is the motoneuron, which essentially acts like a Trojan horse. Indeed, the TeNT molecules first bind to motor cholinergic nerve endings responsible for innervating striated muscles, followed by endocytosis and transport towards the spinal cord (Chapter 20, this volume). Transport studies with $^{125}$I-labeled TeNT reveal a retrograde axonal ascent of TeNT along the motor axons. Indeed, $^{125}$I-TeNT was found in the ventral roots (i.e., containing motor axons) and ventral horn of the spinal cord (i.e., the area containing motoneuron cell bodies). Apparently, α-motoneurons that innervate striated muscle fibers and γ-motoneurons that innervate the muscle spindle can both take up, as well as transport, TeNT. Toxin labeling of the dorsal root, which contains sensory fibers, has also been reported. However, this toxin transport does not seem relevant for the pathophysiology of tetanus because ligature of the dorsal (sensory) root does not prevent labeling of the spinal cord. Moreover, recent studies with a recombinant, fluor-labeled TeNT-fragment reveal binding, uptake, and transport of TeNT in motoneurons, but only binding without transport in dorsal root neurons (Wellhöner, 1992; Halpern and Neale, 1995; Bigalke and Shoer, 2000).

Identification of receptors at motor nerve endings that enable internalization of TeNT, as well as the identity of any facilitating molecule(s) implicated in retroaxonal transport, still remain unresolved. Although not presented here, these issues are fully discussed in Chapter 20, this volume.

TeNT molecules, when retroaxonally transported, arrive at the motoneuron soma and dendrites where they are released into the extracellular space. This transcytosis through motoneurons allows the toxin to bypass the blood-brain barrier and reach the vicinity of nerve terminals that are afferent onto the motoneurons. High TeNT concentrations can be found in particular compartments of the central nervous system during tetanus: for instance, release of one TeNT molecule into a small volume like a synaptic cleft (~1 μm × 1 μm × 50 nm) leads to a "concentration" of ~30 nM! According to *in vitro* experiments, TeNT molecules are recaptured by different types of nerve endings, independent of the type of neurotransmitter molecules they release (Wellhöner, 1992; Bigalke and Shoer, 2000). Consistent with the preferential blocking action of TeNT on GABA and glycine release observed *in vitro*, TeNT suppresses the inhibitory inputs afferent to motoneurons, while the excitatory inputs, which constantly fire motoneurons, are not modified. Therefore, motoneurons are disinhibited and their firing rate increases, inducing sustained muscle contractions that lead to spasms and rigidity. Both the inhibitory descending controls and spinal reflexes that converge onto motoneurons are deeply altered (Figure 19.4). Spindle sensory inputs conveyed by Ia fibers during physiological conditions positively activate the synergistic motoneurons afferent to the same (homonymous) muscle, and simultaneously inhibit motoneurons to antagonize muscles through inhibitory interneurons. Therefore, upon TeNT-induced blockade, or when TeNT blocks the inhibition pathway mediated by Ia interneurons (step 1 in Figure 19.4), the myotactic reflex is deeply altered and leads to co-contraction of agonist and antagonist

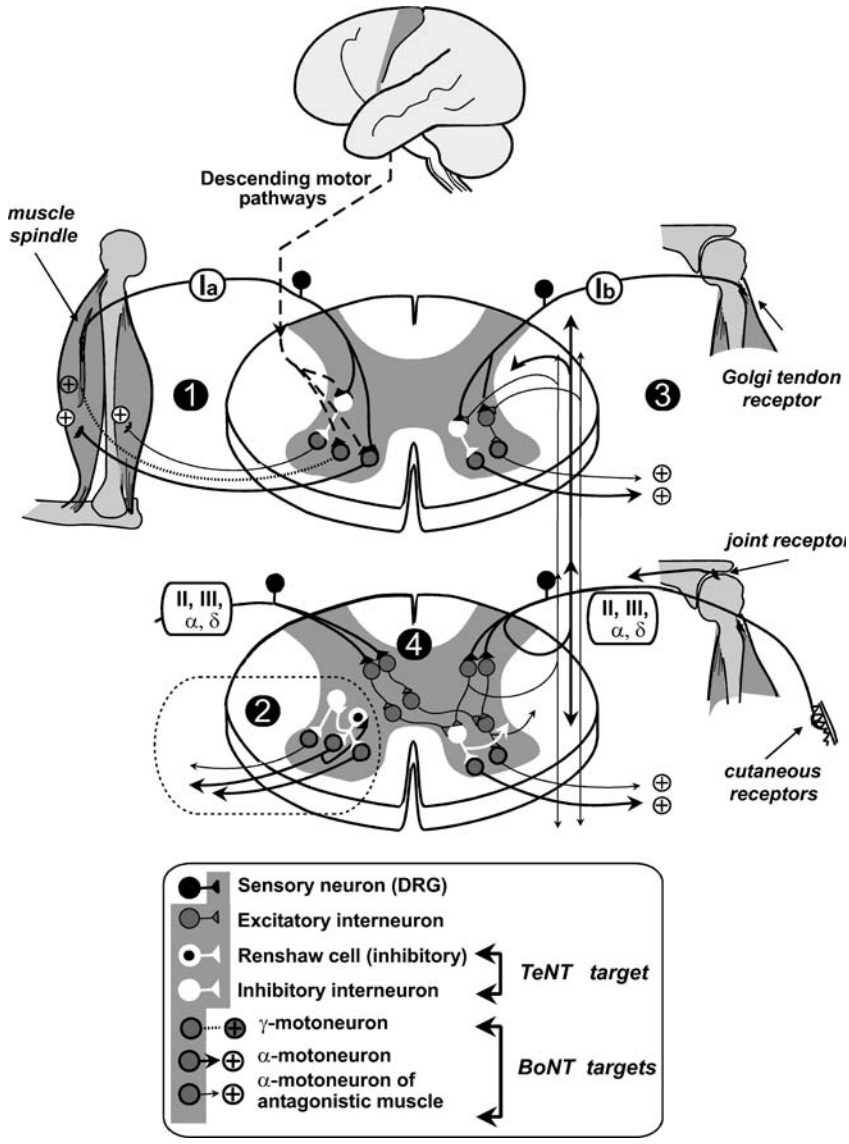



**FIGURE 19.4** Schematic organization of neuronal circuits in the spinal cord. Descending direct or indirect pathways originating from cortical motor areas project onto both motoneurons and interneurons. (1) The neuronal circuit for a myotactic reflex. Ia afferent fibers coming from the muscle spindle excite monosynaptic motoneurons of the homonymous (or synergistic) muscle from which they arise and inhibit neurons to antagonist muscles through an inhibitory neuron. (2) Recurrent inhibition involves the inhibitory Renshaw cells that are excited by collateral branches of motoneurons. Renshaw cells also disinhibit antagonist muscles by inhibiting Ia inhibitory interneurons. (3) The inverse myotactic reflex circuitry. (4) The circuitry for reflexes originating from cutaneous or deep receptors. Note that the latter polysynaptic circuit crosses the spinal cord midline, allowing extension of reflexes to the contralateral side. Moreover, collateral branches allow activation of interneurons in the neighbouring (upper or lower) spinal segments.

Inhibitory neurons (in white) release GABA or glycine, and are the main targets of TeNT. Nerve terminals of α- and γ-motoneurons represent the targets of BoNTs.

muscles working the same joint. TeNT also depresses recurrent inhibition, implicating Renshaw cells (step 2 in Fig. 19.4). Inverse myotactic reflex involves Ib fibers coming from the Golgi tendon organ and participates in a negative feedback system preventing further development of muscle tension upon reaching the desired point. Alteration of this reflex (step 3 in Fig. 19.4) leads to an uncontrolled increase in muscle tension, causing muscle and tendon damage. In a similar way, the many reflexes based on polysynaptic circuits converging on inhibitory interneurons are disorganized (step 4 in Fig. 19.4). Therefore, all local stimuli causing an increase in excitatory input (e.g., sensorial or noxious stimuli) leads to local exacerbation of muscle contractions (Bleck, 1989; Wellhöner, 1992).

The situation is not too different in generalized tetanus. TeNT molecules primarily taken up by motor endings can undergo other transcytotic cycles throughout neurons of second, third, etc. order (Manning *et al*., 1990). Thereby, TeNT entering a single peripheral site and released into the spinal cord disseminates vertically to proximal spinal segments and horizontally to the contralateral side of the spinal cord, thus resulting in progressive muscle rigidity in all muscles. Retroaxonal transport of TeNT along a chain of functionally connected neurons has been exploited to trace the projection pathways between distant brain structures (Cabot *et al*., 1991). Furthermore, to study trafficking pathways and connectivity in sensorimotor circuits, several fusion proteins have been engineered using the atoxic TeNT

C-subunit (TTC) fused to a reporter gene such as *lacZ*, GFP (Green Fluorescent Protein), or EGFP (Enhanced Green Fluorescent Protein) after direct injection of the hybrid proteins (Coen *et al*., 1997; Miana-Mena *et al*., 2002), or expression as a transgene in mice (Maskos *et al*., 2002). When there is a hematogenous portal of entry for TeNT, it enters all motor-nerve terminals and reaches the spinal cord at a number of sites. The most dramatic form of the disease, called *generalized tetanus*, then occurs and is characterized by an overall increase in muscle tone, rigidity, and trismus (Bleck, 1989). Almost all spinal reflexes based on GABAergic and glycinergic transmission are abolished, leading to horizontal and vertical spreading of the response from any stimulus. Therefore, a local stimulus leads to a general response. For example, application of a cutaneous stimulus on a limb can induce profuse contraction of the face, neck, trunk, arms, and legs. When the spasm affects the diaphragm, respiratory failure and death can ensue. Although TeNT injected directly into the brain can induce epilepsy (Benke and Swann, 2004), no central disorder like seizure is observed during tetanus (Bleck, 1989). Therefore, the intrathecal dissemination of TeNT remains largely confined to the spinal cord.

Several other symptoms are detected during generalized tetanus and indicate that TeNT acts also on the autonomous nervous system (e.g., hypertension due to vasomotor spasm, tachycardia, etc.). This hypersympathetic condition is due to disinhibition of the sympathetic reflexes, analogous to that for motoneurons. This is fully consistent with a report that TeNT is also taken up and retrogradely transported in adrenergic neurons. This is followed by toxin delivery to the inhibitory interneurons, whose terminals are afferent to the cell body of adrenergic neurons. Alteration of functions linked to the parasympathetic system has been reported, too (Bleck, 1989; Wellhöner, 1992). All of the above mentioned physical findings observed during local tetanus have a unique origin: TeNT blockade of inhibitory neurotransmitter (GABA and glycine) release.

When the local concentration of TeNT is high, it can also block the release of other neurotransmitters. A high dose of TeNT injected into the lateral rectus muscle of the cat blocks both inhibitory and excitatory synapses simultaneously in abducens neurons (Gonzalez-Forero *et al*., 2003). At the neuromuscular junction (NMJ), TeNT inhibits ACh release, thereby inducing flaccid muscle paralysis *in vitro* (Wellhöner, 1992) and during disease (e.g., cephalic tetanus in humans) (Bleck, 1989). In certain animal species, flaccid motor paralysis may be the only action of the toxin. For instance, application of TeNT into the periphery of goldfish does not produce central actions, but it inhibits ACh release from motor nerve endings. This is consistent with motor terminals being the primary binding targets for TeNT. When captured by motoneuron endings, TeNT is preferentially routed to a transport compartment whose neutral pH prevents translocation into the cytosol of motor nerve terminals. This preferential routing is not absolute, and TeNT appears far less potent than BoNTs in inhibiting ACh release by a factor of 100–1000. This trafficking seems to depend strongly upon the TeNT receptor. Indeed, when the active moiety of TeNT is recombined with the binding domain of BoNT, it acquires a very high efficacy in cholinergic nerve terminals (Wellhöner, 1992).

To summarize, the clinical and experimental findings of tetanus reveal a prominent inhibitory action of TeNT on the release of GABA and glycine. Complexity of the neuronal network and an inherent difficulty in dissociating direct from indirect effects has not allowed identification of other neuronal targets for TeNT. However, the *in vitro* experiments clearly indicate that TeNT blocks release of many, if not all, neurotransmitters.

## Botulism and dissemination of BoNTs within the host

### *Botulism*

Botulism is a severe disease affecting vertebrates, and three main etiological forms are evident in humans. Food-borne botulism has been recognized since the nineteenth century and is induced by ingesting food containing preformed toxin from *C. botulinum* types A, B, E, F, or *C. butyricum*. The second main etiological form of botulism is due to colonization of the gut by *C. botulinum* types A, B, or F, and sometimes neurotoxigenic *C. baratii*. This is the case for infant botulism, which only affects babies up to six months old, and certain cases of gastrointestinal botulism in adults. In addition, like other clostridia (*C. tetani*, *C. perfringens*, etc.), proteolytic strains of *C. botulinum* types A and B can grow quite well in necrotic wounds, which provide appropriate substrate and anaerobic conditions. BoNT is produced *in vivo* at the site of infection, causing "wound-botulism." Note that a "wound" can also occur in the intestinal tract, as this rare form of botulism has been reported in parenteral drug abusers and intranasal cocaine users with sinusitis (Tacket and Rogawski, 1989; Tunkel and Pradhan, 2002).

### *Dissemination of the BoNTs during botulism*

During food-borne botulism, preformed Botulinum complex is ingested with food. Although the several ANTPs associated with BoNTs do not contribute to

poisoning (see below), they seem to play a crucial role in protecting BoNT against acidity and proteases from the stomach. Indeed, oral toxicity of the Botulinum complex is higher than that for BoNTs alone (Ohishi *et al*., 1977; Ohishi and Sakaguchi, 1980). The Botulinum complex has increased helical content in low pH, thus indicating a conformational change (Chen *et al*., 1998). However, the molecular basis for Botulinum complex resistance to low pH and proteases is not yet fully understood. Since type A HA35 is highly resistant to proteolysis by trypsin with or without sugars, this HA may protect Botulinum complex against proteases of the gastrointestinal tract (Fu *et al*., 1997; Sharma *et al*., 1999).

Moreover, HA proteins of the Botulinum complexBotulinum complex seemingly favor BoNT and Botulinum complex adherence to intestinal epithelial cells. Thus, type C Botulinum complex containing HA33, and HA50 binds to microvilli of enterocytes from the upper small intestine of guinea pigs. While HA33 binding is neuraminidase-insensitive, that of HA50 is neuraminidase-sensitive. Type C Botulinum complex containing HA (16S) is much more efficiently absorbed from the intestinal content into the serum than the 12S complex or BoNT/C alone (Fujinaga *et al*., 1997; Fujinaga *et al*., 2000; Fujinaga *et al*., 2004). In human colon carcinoma cells (HT-29), type C 16S Botulinum complex binds to surface glycoproteins like mucin (Nishikawa *et al*., 2004). In addition, local immunization with NTNH and HAs partially protect mice from oral administration of 16S type C or D Botulinum complex (Mahmut *et al*., 2002a). However, in an *in vivo* mouse model, BoNT/A does not require ANTPs for transport from the gastrointestinal tract into the circulatory system (Maksymowych *et al*., 1999). Additionally, in T84 and CaCo-2 human colon carcinoma or Calu-3 human pulmonary cells, ANTPs do not facilitate BoNT/A transcytosis and H chain alone is apparently sufficient for toxin transport through epithelial monolayers (Maksymowych and Simpson, 1998; Park and Simpson, 2003).

When the pH becomes neutral or basic (i.e., when the stomach is buffered by biliary flow in the duodenum, jejunum, and upper intestine), Botulinum complex dissociates and releases BoNT (Simpson, 2004), which then binds to receptors localized on the apical membrane of gut epithelial cells. The BoNT/acceptor complex is endocytosed and directed, via intracellular vesicle trafficking, to the baso-lateral membrane of enterocytes. Here, BoNT is secreted by exocytosis (Maksymowych and Simpson, 1998; Maksymowych *et al*., 1999). This transcytosis allows BoNT, but not the ANTPs, to cross the intestinal barrier and disseminate within the whole organism through the circulatory and lymphatic systems.

While dissemination of the toxin during infant or intestinal botulism is almost identical to that described above for food botulism, there is an interesting difference with wound botulism. Indeed, both BoNTs and ANTPs are produced and found in the extracellular medium. It is not yet documented whether Botulinum complex remains intact during neutral conditions in the extracellular medium, and whether BoNT formed in wounds can undergo hematogenous dissemination. This point deserves further consideration because of its close analogy with the dissemination of small toxin amounts causing side effects when Botulinum complex is injected into tissue for therapeutic purposes (Brin *et al*., 2002). Another point is the recent discovery that proteins associated with BoNT can enhance its action (discussed below).

As all large proteins, BoNTs cannot directly reach the central nervous system. Indeed, tight junctions between endothelial cells of brain and spinal cord capillaries (i.e., the so-called blood-brain barrier) prevent free diffusion of proteins from blood plasma to the cerebro-spinal fluid. Therefore, the action of BoNT is almost restricted to the peripheral nerve system, which includes all of the nerve ganglia.

### *Clinical symptoms and physical findings during botulism and poisoning experiments*

Although dissemination of the BoNT occurs via different routes, there is no clear distinction in symptoms and physical findings displayed by the various forms of botulism (Table 19.1). The clinical picture of botulism often initiates as a dysautonomia, followed by motor paralysis. Initial symptoms of dysautonomia include visual symptoms (blurring, diploplia, and presbytia), followed by dry mouth and constipation. The nausea, vomiting, and diarrhea often reported during food botulism may be due to ill-defined enterotoxins unrelated to BoNT or to action of BoNT on enteric nervous system. Note that during wound botulism, the gastrointestinal symptoms are lacking. Several of the cardiovascular reflexes can be abnormal. Loss of vagal cardiac control, hypothermia, and urinary retention pinpoint an action of BoNT upon the parasympathetic system. Hypotension without reflex tachycardia and depressed vasomotor response to postural changes indicate an action upon the sympathetic nervous system. Dysautonomia induced by BoNT can sometimes be the only manifestation of botulism, and likely corresponds to a benign form of the disease associated with very low amounts of toxin. This is consistent with dysautonomic symptoms, as per side effects observed following therapeutic injection of toxin,

**TABLE 19.1** Physical findings and main characteristics of the blockade of neurotransmitter release induced by clostridial neurotoxins at vertebrate neuromuscular junctions from various animal species

| | BoNT/A | BoNT/E | BoNT/C | BoNT/F | BoNT/D | BoNT/B | TeNT |
|---|---|---|---|---|---|---|---|
| Synaptic target | SNAP-25 | SNAP-25 | SNAP-25, Syntaxin | VAMP | VAMP | VAMP | VAMP |
| **Human** | | | | | | | |
| Neurology | Dysautonomia, flaccid muscle paralysis | Dysautonomia, flaccid muscle paralysis | No case of botulism | Dysautonomia, flaccid muscle paralysis | No case of botulism | Dysautonomia, flaccid muscle paralysis | Spastic muscle paralysis |
| Blockade of evoked quantal release at the NMJ | +++ | ND | +++ | ND | No effect | ND | ND |
| Blockade of spontaneous quantal release at the NMJ | +++ | ND | +++ | ND | No effect | ND | ND |
| **Rat** | | | | | | | |
| Neurology | Flaccid paralysis[1] | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Spasticity |
| Blockade of evoked release at NMJ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| Blockade of spontaneous release at NMJ | +++ | +++ | +++ | +++ | +++ | +/− | No effect[2] |
| **Mouse** | | | | | | | |
| Neurology | Flaccid paralysis[1] | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Spasticity |
| Blockade of evoked release at NMJ | +++ | +++ | +++[3] | +++[3] | +++ | +++ | +++ |
| Blockade of spontaneous release at NMJ | +++ | +++ | +++[3] | +++[3] | +++ | +++ | +++ |
| **Chicken** | | | | | | | |
| Pathology | ND | ND | + | ND | ND | ND | ND |
| Blockade of evoked release | ++ | +++ | ND | ND | ND | ND | ND |
| **Frog** | | | | | | | |
| Neurology | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Flaccid paralysis | Spasticity[3] | Spasticity |
| Blockade of evoked release at NMJ | +++ | +++ | +++ | +++ | +++ | No effect | No effect[3] |
| Blockade of spontaneous release at NMJ | +++ | +++ | +++ | +++ | +++ | ND | ND |
| **Fish (Goldfish, Torpedo)** | | | | | | | |
| Neurology | | | | | | | Flaccid Paralysis |
| Blockade of evoked and spontaneous release at cholinergic synapses | +++ | ND | ND | ND | ND | ND | +++ |

Subjective gradation: very potent (+++), less effective (++, +), variable effects (+/−). In some cases, toxin action has not been determined (ND). For further discussion and references see Molgo *et al.*, 1990 or Humeau *et al.*, 2000.

[1] BoNT/A-induced paralysis is less marked on juvenile as compared to adults (Bambrick and Gordon, 1989; Ma *et al.*, 2002)

[2] but TeNT blocks spontaneous quantal events in the central nervous system of the rat (Capogna *et al.*, 1997).

[3] No effect at the NMJ from animals having central effects (J. Molgo, unpublished observation).

which correspond to hematogenous dissemination of minute amounts of BoNT from the injection site (Dressler and Benecke, 2003).

The many symptoms of autonomic dysfunction during botulism are consistent with BoNTs acting upon both cholinergic and catecholaminergic neurotransmission. Effects of BoNTs are likely exerted at pre- and post-ganglionic levels, since ganglions are not insulated by the blood-brain barrier. Gastrointestinal troubles observed during foodborne and infant, but not wound, botulism suggest that BoNT can directly (without hematogenous dissemination) affect neurons of the enteric nervous system. This action has promoted BoNT use for treating overactive smooth muscles and sphincters, such as the lower esophageal sphincter for esophageal achalasia, internal anal sphincter for anal fissures, or secretory disorders like drooling and hyperhydrosis (Tscheng, 2002; Brisinda *et al*., 2004; Cordivari *et al*., 2004; Naumann and Jost, 2004).

The second series of manifestations during botulism consists of symmetric weakness or descending paralysis. Observation of this symptom prompts the clinician to diagnose botulism. However, confusion in making a diagnosis as Guillain-Barre or myasthenic syndrome may occur, but botulism is characterized by an absence of sensory symptoms. Flaccid paralysis clearly indicates BoNT effects on the motor system, which initially affects striated muscles innervated by cranial nerves producing ptosis, followed by a depressed gag reflex, dysphagia, dysarthria, facial paralysis, tongue weakness, and several neuro-ophthalmological signs. After these early symptoms, general paralysis extends from proximal, to distal, muscles of the neck and limbs. The deep tendon reflex is strongly depressed. When the diaphragm is attacked, respiration is depressed and the need for mechanical ventilation becomes imperative (Tacket and Rogawski, 1989; Low, 2002).

Overall, the clinical picture of botulism indicates that flaccid neuromuscular paralysis is due to selective inhibition of neurotransmission between α-motoneurons and striated muscle fibers. This paralytic action of the BoNTs is now widely exploited in therapeutics (Brin *et al*., 2002; Turton *et al*., 2002; and Chapter 58 of this volume).

Experiments performed at the NMJ reveal that following application of BoNTs to isolated nerve-muscle preparations, the postsynaptic responses (i.e., endplate potentials resulting from nerve stimulation) have amplitudes insufficient to reach the threshold for an action potential in muscle, thereby causing paralysis (Table 19.2) (Habermann and Dreyer, 1986; Simpson, 1989; Molgo *et al*., 1990; Van der Kloot and Molgó 1994). An approximately 20% decrease in the endplate potential amplitude sufficiently prevents muscle fiber contraction. Asphyxia occurs when fewer than 10% of the diaphragm fibers are paralyzed. The most dramatic form of botulism follows ingestion of ~0.1 to 1 μg BoNT. Therefore, the circulating/extracellular BoNT concentrations causing death (<<0.001 nM) are below those inducing muscle paralysis *in vitro* (~0.01–0.1 nM), and far from those (1–10 nM) required to induce a nearly complete inhibition of ACh release from nerve terminals (Molgo *et al*., 1990; Poulain *et al*., 1996). BoNT also blocks transmission between the γ-motoneurons and the muscle spindle, albeit with different kinetics as compared to the motor endplate (Filippi *et al*., 1993; Rosales *et al*., 1996). This latter action of BoNTs alters the spinal reflexes initiating from the muscle spindle via the sensory Ia fibers.

*In vitro* studies also revealed that BoNTs prevent release of various neurotransmitters other than ACh, such as adrenaline, noradrenaline, dopamine, ATP, glutamate, aspartate, GABA, and neuropeptides like substance P or CGRP (Habermann and Dreyer, 1986; Wellhöner, 1992; Bigalke and Shoer, 2000; Humeau *et al*., 2000; Purkiss *et al*., 2000; Welch *et al*., 2000; Foran *et al*., 2003; Durham *et al*., 2004). To summarize, like TeNT, none of the BoNTs are specific for a given neurotransmitter and thus cannot be considered as only "anti-cholinergic" neurotoxins.

**TABLE 19.2** Steps of neuromuscular transmission altered by botulinum neurotoxins (BoNTs), after local injection

| Steps of neuromuscular transmission | Effect of BoNTs |
|---|---|
| Action potential at nerve endings | Unchanged |
| • $Ca^{2+}$ influx | Unchanged |
| • ACh exocytosis | Inhibited |
| • Responsiveness of postsynaptic AChRs | Enhanced by the increased number of AChRs and changed properties |
| • End Plate Potential (EPP) | Overall, it is diminished* |
| • Muscle action potential | Abolished if subthreshold EPP. Change in the α-subunit of Na channels ($Na_V1.4$ to $Na_V1.5$) |
| Nerve evoked single muscle fiber contraction | Abolished if subthreshold EPP |
| • Nerve evoked muscle twitch | Gradually diminished, but non-linearly correlated with inhibited ACh-release |

*Due to fast muscle fiber atrophy, the membrane impedance (input resistance and capacitance) increases in muscle fibers. During moderate poisoning, this can balance the decrease in ACh release, leading to unchanged EPPs.

### *Do BoNTs directly affect the central nervous system during botulism or after peripheral application?*

Very few cases of botulism associated with pyramidal signs have been reported (Santini *et al.*, 1999). Motoneuron disinhibition after intramuscular injection of Botulinum complex/A, and changes in the reciprocal inhibition after injection of Botulinum complex/A for therapeutic purposes, have been reported. Injection of Botulinum complex/A into cat extraocular muscles changes the discharge patterns of motoneurons innervating both the ipsi- and contra-lateral eye muscles (Moreno-Lopez *et al.*, 1997). Morphological alterations in the synaptic organization surrounding motoneurons (Pastor *et al.*, 1997) and several changes in the motor system (brainstem and cortical levels) have also been reported (Curra *et al.*, 2004). One cannot exclude that these central actions are indirect, resulting from alterations of the sensorimotor integration through a direct action of BoNT on peripheral sensory mechanisms (i.e., the action of BoNT on the cholinergic innervation of the muscle spindle). Several of the brain stem and cortical changes reported after administration of BoNTs to patients are likely indirect (Curra *et al.*, 2004). In opposition to this theory, it is conceivable that BoNTs enter the central nervous system at a few sites where the blood-brain barrier is discontinuous, due to fenestrated capillaries (e.g., the portal vasculature in the hypophysis and pituitary stalk). This may explain some of the autonomic symptoms, since hypothalamic neurons are at a short diffusional distance from the hypophysal portal system. In fact, the main possibility that deserves consideration is transcytosis of BoNT/A throughout the motoneuron, as this does occur for TeNT. Indeed, retroaxonal ascent of $^{125}$I-BoNT/A up to the motoneuron bodies has been well documented (Wellhöner, 1992). Are BoNTs secreted by motoneurons and do these molecules disseminate into the spinal tissue, thus altering synaptic transmission? The BoNTs can alter synaptic transmission in the central nervous system, because their direct administration into the spinal cord or brain (Luvisetto *et al.*, 2003), brain slices, or cultured neurons blocks release of various neurotransmitters (Wellhöner, 1992; Humeau *et al.*, 2000).

### *Characteristics of BoNTs and TeNT blocking action on synaptic transmission in* ex vivo *or* in vitro *experiments*

The main action of BoNTs or TeNT on neurotransmitter release evoked by nerve action potential dramatically reduces the postsynaptic amplitude response to practically nil (Table 19.2) (Habermann and Dreyer, 1986; Simpson, 1989; Molgo *et al.*, 1990; Wellhöner, 1992; Humeau *et al.*, 2000).

Propagation of the nerve action potential at the nerve terminal, and the ensuing $Ca^{2+}$ influx across the presynaptic membrane (Table 19.3), are not significantly altered by BoNTs or TeNT. Overall, postsynaptic cells respond normally to applied neurotransmitter, and no obvious structural abnormalities or degenerative changes have been observed that account for inhibited synaptic transmission. With BoNT/C, a subtle disorganization of active zones has been reported (Stanley *et al.*, 2003). A high dose of TeNT causes retraction of presynaptic inhibitory buttons and postsynaptic spines when the local concentration of TeNT in the central nervous system is high. This is an indirect effect, due to the silencing of postsynaptic neurons after inhibition of both inhibitory and excitatory inputs (Gonzalez-Forero *et al.*, 2004). Therefore, neurotransmission inhibited by TeNT or the BoNTs is attributable to reduced numbers of synaptic vesicles fusing with the plasma membrane following nerve stimuli. However, the BoNTs and TeNT do not inhibit nonvesicular release of compounds like nitric oxide (NO) that diffuse (Morris *et al.*, 2001), or are transported (McMahon et al., 1992), across the plasma membrane.

Spontaneous exocytosis of transmitter, which gives rise to spontaneous miniature responses, is almost completely abolished after exposure to the BoNTs or

**TABLE 19.3** Further characteristics of the synaptic actions induced by BoNTs and TeNT

| Neurotoxins | BoNT/A | BoNT/E | BoNT/C | BoNT/B, D, F, and TeNT |
|---|---|---|---|---|
| Synaptic target | SNAP 25 | SNAP-25 | SNAP-25 & Syntaxin | VAMP/synaptobrevin |
| Quantal dispersion during evoked neurotransmitter release | Synchronous | Synchronous | Synchronous[1] | Asynchronous |
| Morphological docking | No effect or slightly increased | ND | No effect or slightly disturbed | No effect or slightly increased |
| $Ca^{2+}$ influx | No effect | ND | No effect but, (see text) | No effect |

ND: not determined. For references see text and Molgo *et al.*, 1990; Poulain *et al.*, 1995; Humeau *et al.*, 2000.
[1]J. Molgo, unpublished observation

TeNT (Habermann and Dreyer, 1986; Simpson, 1989; Molgo *et al.*, 1990; Humeau *et al.*, 2000). Moreover, BoNT/A can block quantal transmitter release from certain glial cells like astrocytes (Verderio *et al.*, 1999; Araque *et al.*, 2000; Abdipranoto *et al.*, 2003). The number of synaptic vesicles docked at the presynaptic membrane appears elevated versus untreated terminals. The latter findings pinpoint TeNT and BoNTs action downstream from docking of synaptic vesicles at the active zones, possibly during the fusion process.

Several electrophysiological studies have reported decreased amplitude and an increased time course for the elementary postsynaptic responses (the so-called "quanta") elicited by the exocytosis of a single synaptic vesicle. This change seems more pronounced for BoNT/A than for the other neurotoxins. These effects superpose to the above described toxin actions and appear to have both pre- and post-synaptic origins. Although the synaptic vesicle contents are unchanged because BoNTs and TeNT affect neither the synthesis nor storage of neurotransmitter, it is conceivable that the quantity of ACh delivered after synaptic vesicle fusion is diminished because the time period for opening of the fusion pore is shorter. This possibility is consistent with the notion that neurotransmitter release may implicate transient opening of a fusion pore, as postulated in the "Kiss and Run" model for transmitter release. However, there has been no detectable change in the fusion pore openings of chromaffin cells (Graham *et al.*, 2000). On long-lasting experiments (e.g., *in vivo* poisoning followed by *ex vivo* examination), postsynaptic changes in receptor densities and conductance are also likely. Therefore, small amplitude and slow elementary synaptic responses might be an indirect consequence of the synaptic remodeling produced by the toxins.

The blockage induced by TeNT and BoNT/B, D, and F exhibits several features that make it clearly distinct from that induced by BoNT/A, C, and E, as revealed by *ex vivo* examination of NMJs poisoned *in vivo* (Tables 19.3 and 19.4). This gross distinction correlates with identification of toxin targets (see below). For example, the few synaptic vesicle fusions triggered by BoNT/A, C, or E at nerve terminals remain synchronized, whereas those occurring after TeNT or BoNT/D and F are characterized by a temporal dispersion. Any treatment aimed at increasing the release probability of BoNT/A involves: (i) raising extracellular $Ca^{2+}$; (ii) tetanic-nerve stimulation; and (iii) enhancing phasic $Ca^{2+}$ influx, thus increasing the number of synaptic vesicles that fuse upon arrival of the action potential at the nerve endings, provided that transmitter release is not fully blocked. Under these conditions, a recovery of synaptic responses may occur with amplitudes sufficient to depolarize the membrane over the threshold needed for generating a muscle action potential and twitching. In contrast, at TeNT-, BoNT/B-, D-, or F-poisoned NMJs, these treatments have a very low efficacy (Table 19.4) since the remaining asynchrony of release events prevents build-up of postsynaptic responses large enough to allow transmission recovery (Molgo *et al.*, 1990; Humeau *et al.*, 2000; Szule and Coorssen, 2003).

Similar distinctive actions between the various BoNT serotypes or TeNT were revealed following α-latrotoxin (α-LTX) induction of synaptic vesicle fusion at BoNT- or TeNT-poisoned NMJs (Table 19.4). Indeed, at normal NMJs, black widow spider venom or its major component, α-LTX, induces an asynchronous release of ACh quanta that exhausts the synaptic vesicles contained in the nerve terminals (Van der Kloot and Molgó, 1994; Schiavo *et al.*, 2000). Black widow spider venom or α-LTX also stimulates vesicle fusion at BoNT/A-poisoned nerve terminals that leads to exhaustion of the existing synaptic vesicle pool, whereas this venom/toxin has only moderate action upon BoNT/B- or TeNT-treated NMJs.

**TABLE 19.4** Pharmacological manipulations that recover neurotransmitter release from nerve terminals blocked by clostridial neurotoxins

| | **BoNT/A** | **BoNT/E** | **BoNT/C** | **BoNT/B, D, F, and TeNT** |
|---|---|---|---|---|
| Synaptic target | SNAP 25 | SNAP-25 | SNAP-25 & Syntaxin | VAMP |
| Potentiation of $Ca^{2+}$ influx | ++ | + | ++ | + |
| Aminopyridines | +++ | +/– to +++ | ND | + |
| $Ca^{2+}$ ionophores | ++ | ND | ++ | ++ |
| $La^{3+}$, $Zn^{2+}$, $Sr^{2+}$ | +++ | ++ | ++ | + |
| α-latrotoxin or black widow spider venom | +++ | ND | +++ | + |

Subjective gradation for reversing neurotoxin actions: +++, very potent; ++, +, less effective; +/–, variable effects; ND, not determined. For references, see text and Molgo *et al.*, 1990; Poulain *et al.*, 1995; Humeau *et al.*, 2000.

## The molecular mechanisms of action for clostridial neurotoxins

### *BoNTs and TeNT specifically cleave VAMP, SNAP-25, or syntaxin in nerve terminals*

The light chain of BoNTs and TeNT inhibits transmitter release via $Zn^{2+}$-dependent, endopeptidase activity, which *in vitro* requires reduction of the disulfide interchain bridge linking the heavy and light chains (Herreros *et al.*, 1999; Schiavo *et al.*, 2000; Turton *et al.*, 2002; Lalli *et al.*, 2003; Simpson, 2004).

Three synaptic proteins have been originally identified in mammalian nerve terminals as the proteolytic targets for clostridial neurotoxins. Namely, these are VAMP-2 (Table 19.5), which is cleaved by TeNT, BoNT/B, D, F and G; SNAP-25 (Table 19.6), which is attacked by BoNT/A, C, and E; as well as syntaxin-1 (Table 19.7), which is exclusively proteolyzed by BoNT/C. As discussed above, these three proteins are the SNAREs involved in synaptic vesicle fusion with the plasma membrane. Experimental findings that demonstrate proteolytic inhibition of neuroexocytosis are discussed later.

Several closely related homologues of the SNAREs are also cleaved by the clostridial neurotoxins (Tables 19.5–7). It is beyond the scope of this chapter to review all the SNAREs cleaved by these toxins. Their susceptibility to cleavage may display marked differences, as compared to proteolysis of the three original SNAREs. Mouse SNAP-23 is cleaved by BoNT/E, but to a reduced extent by BoNT/A. Mouse VAMP-1 (also present in nerve terminals), -2, and -3 are cleaved by TeNT and some BoNTs with similar efficacy. Syntaxins-2 and -3, but not syntaxin-4, are cleaved by BoNT/C. The main reasons for differences in proteolytic susceptibility are point mutations at the cleavage site or flanking regions. Variations in the amino acid sequence of SNAREs have occurred throughout time and subsequently made several animal species resistant, or partially resistant, to subsets of the clostridial neurotoxins (Tables 19.5–7). For example, rat VAMP-1 resists proteolysis by either TeNT or BoNT/B due to point mutations at the cleavage site. Leech SNAP-25 cannot be cleaved by BoNT/A due to a mutation at the dipeptide site attacked by the toxin. Human SNAP-23 can neither be cleaved by BoNT/A nor BoNT/E, and the resistance towards BoNT/E is due to an amino acid exchange within a region flanking the cleavage site. In general, most toxin-insensitive VAMPs are generated by mutations within the cleavage site (Table 19.5), while toxin-resistant isoforms of SNAP-25, or related analogues, result from amino acid substitutions in and around this site (Humeau *et al.*, 2000). Keep in mind that resistance to the BoNTs also results from a lack of receptors on nerve terminals, as demonstrated by the inability of BoNT/D and BoNT/B to alter ACh release at human and frog NMJs, respectively (Coffield *et al.*, 1997; Coffield *et al.*, 1999).

### *How do BoNTs and TeNT recognize specifically the SNAREs*

Selectivity of the clostridial neurotoxins for their substrate reportedly arises from a highly conserved recognition domain, distinct from the cleavage site, that is specific to the SNARE proteins (Rossetto *et al.*, 1994; Schiavo *et al.*, 2000). Furthermore, TeNT exploits a second exosite, adjacent to the cleavage site (Cornille *et al.*, 1997). Changes in the amino acid sequence at the recognition site affect cleavage of SNARE by a toxin. For example, the splice variant SNAP-25a expressed during early development is partially resistant to BoNT/A, but not BoNT/E (Puffer *et al.*, 2001). The amino acid sequence around the cleavage site is identical in both SNAP-25a and b isoforms, but the N-termi-

**TABLE 19.5** Clostridial neurotoxin cleavage sites in VAMP/synaptobrevin

| VAMP isoform | Vertebrate species | Specific cleavage sites | Resistance to cleavage by |
|---|---|---|---|
| | | BoNT/F BoNT/D TeNT & BoNT/B BoNT/G | |
| VAMP-1 | Human, louse, bovine, torpedo | —dkvlerd**qk**l**s**elddradalqagas**q**f**e**ss**aa**klkrkyww— | None |
| VAMP-1 | Rat, chicken | —dkvlerd**qk**l**s**elddradalqagas**vf**ess**aa**klkrkyww— | TeNT and BoNT/B |
| VAMP-2 | Human, mouse, rat, bovine, chicken | —dkvlerd**qk**l**s**elddradalqagas**q**f**e**t**s**aaklkrkyww— | None |
| VAMP-3 (Cellubrevin) | Human, mouse, rat, chicken | —dkvlerd**qk**l**s**elddradalqagas**q**f**e**t**s**aaklkrkyww— | None |
| VAMP-7 (TI-VAMP) | Rat | —dlvaqrg**er**l**e**llidktenlvdssv**tf**ktt**sr**nlaramcm— | TeNT and all BoNTs |

Black boxes denote mutations at the cleavage sites. Gray boxes denote mutations around cleavage sites.

**TABLE 19.6** Clostridial neurotoxin cleavage sites in vertebrate SNAP-25 and SNAP-23

| SNAP isoform | Vertebrate species | Specific cleavage sites | Resistance to cleavage by |
|---|---|---|---|
| | | BoNT/E ↓ BoNT/A BoNT/C ↓↓ | |
| SNAP-25a & SNAP-25b | Human, mouse, rat | —qnrqid**ri**mekadsnktrideanq**ra**tkmlgsg$^{end}$ | None |
| SNAP-25 (1) | Chicken | —qnrqid**ri**meklipikpglmkpt**svq**qrcsavvk$^{end}$ | **BoNT/A & C**[*] |
| SNAP-25 (2) | Chicken | —qnrqid**ri**mekadsnktrideanq**ra**tkmlgsg$^{end}$ | None |
| SNAP-25 A | Goldfish | —qnrqid**ri**mdmadsnktrideanq**ra**tkmlgsg$^{end}$ | None[*] |
| SNAP-25 B | Goldfish | —qnrqid**ri**mekadsnktrideanq**ra**tkmlgsg$^{end}$ | None[*] |
| SNAP-25 | Zebrafish | —qnrqie**ri**qskavvnesrineanq**ra**tklisr$^{end}$ | **BoNT/E**[*] |
| SNAP-25 | Torpedo | —qnaqvd**ri**vvkgdmnkarideank**ha**tkml$^{end}$ | **BoNT/E, A, C**[*] |
| SNAP-23 | Human | —qnpqik**ri**tdkadtnrdridiana**ra**kklids$^{end}$ | **All** |
| SNAP-23 (Syndet) | Mouse, rat | —qnqqig**K**itekadtnknridiant**ra**kklids$^{end}$ | **BoNT/A & C** |
| SNAP-23 | Chicken | —qnkqid**ri**nvkadtnrdr**i**eqanir**a**kklidn$^{end}$ | **BoNT/A & C**[*] |

Black boxes denote mutations at the cleavage sites. Gray boxes denote mutations around cleavage sites.
[*]Expected

nal half that contains three of the four recognition sites differs and thus leads to partial proteolytic resistance of SNAP-25a (Puffer *et al.*, 2001).

### *Do ANTPs enhance the proteolytic activity of BoNTs within nerve terminals?*

Unexpectedly, the ANTPs probably play a role in the endopeptidase activity of BoNTs. Thus, type A Botulinum complex is more active (15–17 fold) than pure BoNT/A under reducing or non-reducing conditions *in vitro* and *in vivo* (Cai *et al.*, 1999). Among ANTPs, type A HA35 enhances 21–25 fold the proteolytic cleavage of SNAP-25 by BoNT/A or BoNT/E *in vitro* under non-reducing conditions, and by 13-fold in synaptosomes (Sharma and Singh, 2004). HA35 does not reduce the disulfide interchain bridge, which is not required for activity. HA35 strongly binds to BoNT/A and likely exposes the enzymatic site in an active conformation for substrate without opening the belt formed by Hn. This is consistent with the molten globule structure of BoNT/A. HA35 also binds to synaptotagmins, which are proposed receptors for the BoNTs (see Chapter 20, this volume), and possibly enters synaptosomes in support of subsequent enhancements of intracellular activity for BoNT/A (Sharma and Singh, 2004). This novel possibility has to be carefully considered when toxin complex is injected for therapeutic purposes.

**TABLE 19.7** Botulinum toxin C cleavage sites in vertebrate syntaxins

| Syntaxin isoform | Vertebrate species | Cleavage site for BoNT/C ↓ | Resistance to cleavage by |
|---|---|---|---|
| Syntaxin 1A | Human, bovine, mouse, rat | $^{245}$eravsdtk**ka**vkyqskar$^{262}$ | No |
| Syntaxin 1B | | $^{244}$eravsdtk**ka**vkyqskar$^{261}$ | No |
| Syntaxin 1B | Chicken | $^{238}$vpevfvtk**S**avmyqcksr$^{255}$ | ? |
| Syntaxin 2 | Rat | $^{245}$ehakeetk**ka**ikyqskar$^{262}$ | No |
| Syntaxin 3 | Rat | $^{244}$ekardetr**ka**mkyqgqar$^{261}$ | No |
| Syntaxin 4 | Rat | $^{244}$ergqehvk**l**alenqkkar$^{261}$ | Yes |

Black boxes denote mutations at the cleavage sites. Gray boxes denote mutations around cleavage sites.
[*]Expected

### *Physiology of BoNTs and TeNT access to the SNAREs in living nerve terminals*

With regard to their low $k_{cat}$ and Michaelis constant $K_m$, TeNT, and BoNTs are rather poor enzymes (Li *et al.*, 2000). This is surprising for toxins that are considered the most potent blockers known of synaptic function. At *Aplysia* synapses, the blockage of ACh release by an intracellular concentration of 10 nM of TeNT, BoNT/A, or BoNT/B starts with a delay of only a few (4–10) minutes after the intracellular injection of toxin (Poulain *et al.*, 1996). Considering that the mean diameter of *Aplysia* nerve terminals is around 2.5 μm, the nerve ending volume is about 16 femtoliter, and a 10 nM concentration corresponds to less than 100 L chain molecules per button. This represents very few molecules as compared to the 50–80 $\times$ $10^3$ synaptic vesicles (each decorated with approximately 10 VAMP molecules) and the 400–600 release sites present in each nerve terminal (Humeau *et al.*, 2002). Therefore, the very short delay between the clostridial neurotoxin injection and blockage (Poulain *et al.*, 1996) suggests that the toxin molecules are targeted to key sites where proteolytic action has immediate consequences upon exocytosis.

A mechanism that likely accelerates neurotransmitter inhibition is the prepositioning of L chain near the release site. Indeed, BoNT/A-L chain contains a sequence within the N-terminus that facilitates plasma membrane localization and concentration near the fusion sites (Fernandez-Salas *et al.*, 2004).

A second important factor determining SNARE attack by the different L chains involves the quaternary structure. Since the BoNTs and TeNT bind to their targets via at least two distant sites (i.e., those involved in cleavage and recognition), the accessibility of substrate cleavage into the catalytic cleft in the toxin L chain is dictated by the interaction of SNAREs with partner proteins. Biochemical evidence demonstrates that SNARE proteins are protected from proteolytic attack when bound in a ternary complex (Hayashi *et al.*, 1994). Structural studies reveal masking of cleavage sites and/or recognition motifs within the SNARE complex core (Sutton *et al.*, 1998). Therefore, it is likely that when the four-helix bundle is formed (i.e., when synaptic vesicles are docked at the fusion sites), the clostridial neurotoxins cannot attack their respective SNAREs. When SNAP-25 forms a dimer with syntaxin-1, it is also protected against BoNT/A (Margittai *et al.*, 1999).

On the contrary, certain protein-protein interactions are not protective: SNAP-25 bound to synaptotagmin can be cleaved by either BoNT/A or E (Schiavo *et al.*, 2000). When VAMP forms a dimer or associates with synaptophysin, it also becomes substrate for cleavage (Reisinger *et al.*, 2004). Thus, a possible rule is that when the SNARE domains involved in SNARE-partner interactions do not comprise the recognition motif or cleavage site, the SNARE can be proteolyzed. The physiological relevance of this proposal is indicated by several functional studies. For example, consistent with the notion that Rab3 acts as an upstream regulator of the Rim/Munc-13/Munc-18/syntaxin/SNAP-25 cascade, which regulates transition of SNAREs between open and closed configurations at the release site, intraneuronal application of a Rab3 dominant negative mutant delays blocked release induced by BoNT/A, BoNT/B, and TeNT on *Aplysia* synapses (Johannes *et al.*, 1996). $Ca^{2+}$-calmodulin binds to VAMP, thereby impeding its cleavage by TeNT but not BoNT/B, which uses another recognition site. Indeed, the VAMP domain that binds $Ca^{2+}$-calmodulin is located immediately adjacent to the C-terminal side of the peptide bond cleaved by TeNT (Quetglas *et al.*, 2000; de Haro *et al.*, 2004) and overlaps with the second recognition exosite exploited by TeNT (Cornille *et al.*, 1997). Obviously, this provides a rationale for the observation that VAMP cleavage by TeNT, but not BoNT/B, is nerve terminal-activity dependent (Hua and Charlton, 1999). In the same line, several experimental findings suggest that other environmental conditions modify cleavage accessibility of the SNAREs. For example, the blockage kinetics of intracellularly applied BoNT/B or TeNT, but not BoNT/A, are strongly temperature dependent (Poulain *et al.*, 1996). Interactions of the SNAREs with the lipid environment likely condition their folding and modify their accessibility for cleavage. Indeed, the proteolytic activity of BoNT/A-L chain is higher when SNAP-25 is inserted into membranes or liposomes containing negatively charged phospholipids versus soluble forms of SNAP-25. Possibly, the membrane conformation of SNAP-25 is better recognized by the toxin, or phospholipids facilitate an electrostatic toxin-substrate interaction (Caccin *et al.*, 2003). A similar role for a lipid environment has also been suggested for the cleavage of VAMP by TeNT (de Haro *et al.*, 2004). Altogether, these biochemical and functional observations indicate that the recognition domains and cleavage sites of the various SNAREs are sequestered under the same physiological circumstances. In other words, toxins likely access their substrate only during defined "physiological windows" (Figure 19.5, top panel), which do not necessarily coincide for all toxins. The open configurations of the SNAREs are acquired mainly during the late exocytotic events. Therefore, we speculate that the "windows" during which the toxins have easy access to their targets are open when synaptic vesicles tether and dock at the active zones, which is also when the SNAREs dissociate from their

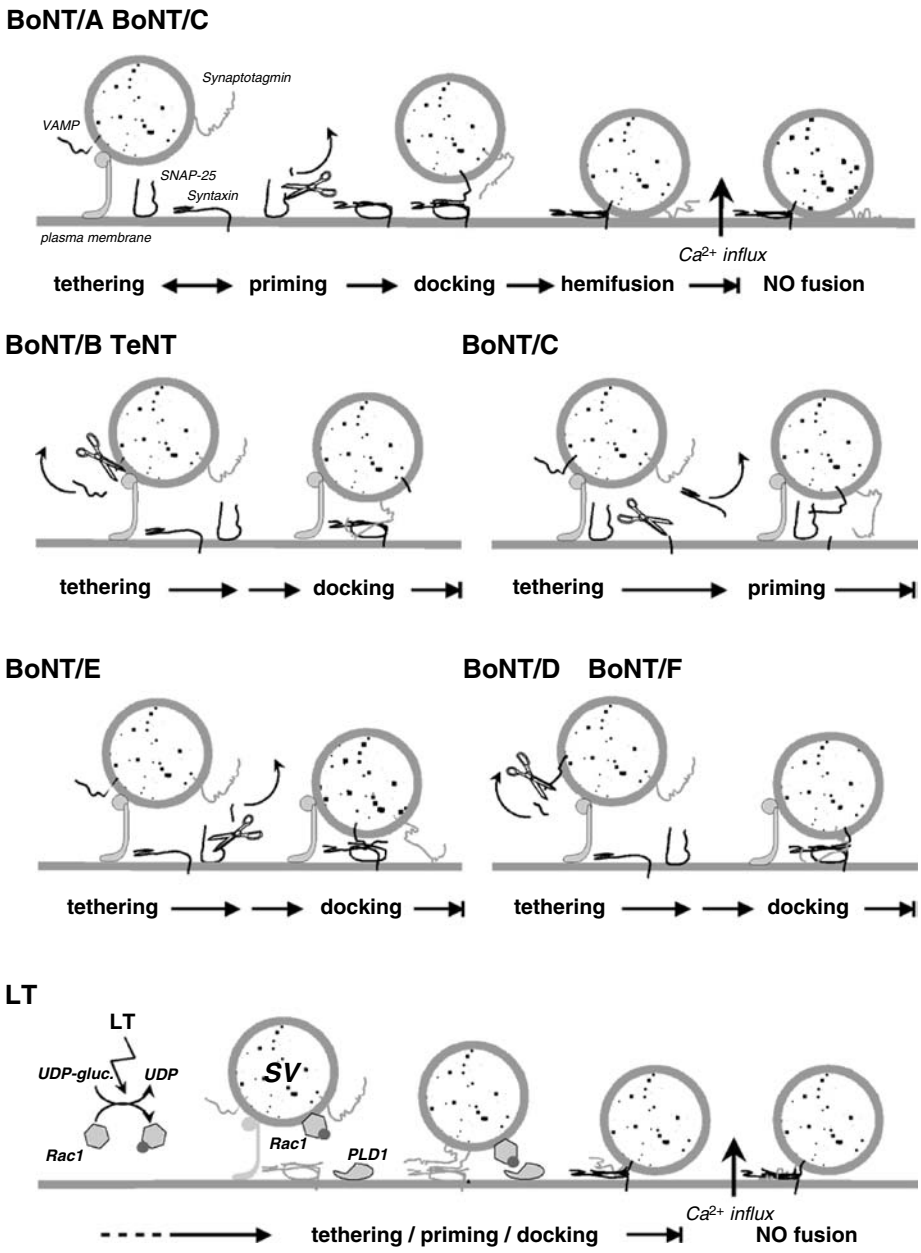



**FIGURE 19.5** Models for the inhibitory action of BoNTs, TeNT, and LT on exocytosis. During the priming events that precede assembly of the SNAREs into a complex, an open configuration is adopted by the SNAREs (VAMP, SNAP-25, or syntaxin). This defines a "physiological window" during which TeNT or the various BoNTs have free access to the respective cleavage site. A commonality of all the BoNTs and TeNT is that the peptide released into the cytosol, following target proteolysis, is likely to diffuse from the exocytosis sites (as denoted by a curved arrow). Therefore, assembly of intact and truncated SNARE into complex may be impossible (BoNT/C acting on syntaxin, BoNT/B, TeNT), altered (BoNT/E, BoNT/D, BoNT/F), or complete (BoNT/A, BoNT/C acting on SNAP-25). Therefore, only a limited number of steps can occur during the sequence of events that lead to membrane fusion (compare with Fig.19. 1). In all cases, the process stops before fusion.

The action of LT is quite different, while converging upon disruption of the fusion step. Glucosylated, vesicle-bound Rac cannot stimulate PLD and ensuing production of fusogenic phospatidic acid, which is necessary for membrane fusion (compare with Fig.19. 2).

chaperones and have not yet assembled into the fusogenic SNARE complex.

To summarize, prepositioning of toxin L chains near the release site enables toxin attack upon their protein targets during the defined "physiological window," which mainly corresponds to the molecular events preceding exocytosis by a few seconds. Thus, the toxins likely affect the small pool of synaptic vesicles undergoing exocytosis, leading to immediate physiological consequences. This probably amplifies toxin efficacy and provides a rational explanation for the paradox that TeNT and BoNTs are poor enzymes, yet they quite effectively block synaptic function.

### *Relationship between SNARE cleavage and blockade of neurotransmitter release*

*Blockade induced by SNAP-25 cleavage*

The clear-cut demonstration that cleavage of SNAP-25 blocks BoNT/A action was first provided by elegant studies with the leech synapse. Indeed, leech SNAP-25 cannot be cleaved by BoNT/A, and accordingly cultured Retzius cells are resistant to BoNT/A. However, the sensitivity of serotonin release to BoNT/A action

could be conferred by replacing amino acids 196–209 of leech SNAP-25 with those from the rat protein (Bruns *et al.*, 1997). A further, albeit indirect, demonstration of the causal link between SNAP-25 cleavage and exocytosis blockade was obtained with insulin secreting cells. Human SNAP-23 can replace SNAP-25 in SNARE complexes, and due to BoNT/A resistance, overexpression rescues insulin secretion after blockade by BoNT/A (Sadoul *et al.*, 1997), although SNAP-23 is less efficient than SNAP-25 in mediating secretion (Sorensen *et al.*, 2003). In cultured rat hippocampal neurons intoxicated with BoNT/A or BoNT/E, only glutamatergic synapses are blocked, whereas GABAergic synapses show exo-/endo-cytotic activity. This correlates with different expression levels of SNAP-25. Relative to glutamatergic neurons that express SNAP-25, GABAergic neurons lack SNAP-25. Possibly, SNAP-25 is replaced by SNAP-23 or other unidentified homologues of BoNT-insensitive SNAP (Verderio *et al.*, 2004). Expression in chromaffin cells of mutated SNAP-25 lacking the last nine amino acids reduces secretion to a similar extent as BoNT/A (Wei *et al.*, 2000). Expression of mutated SNAP-25 not cleaved by BoNT/A allows full recovery of secretory activity (O'Sullivan *et al.*, 1999). SNAP-25 immunoreactivity decreases when synaptic inhibition occurs at both central synapses and at the NMJs, albeit this correlation is not strict (Capogna *et al.*, 1997; Kalandakanond and Coffield, 2001a; Kalandakanond and Coffield, 2001b; Keller and Neale, 2001). Several findings are still puzzling; although *Torpedo* SNAP-25 is not cleaved by BoNT/A *in vitro* (Washbourne *et al.*, 1997), this toxin abolishes ACh release from the *Torpedo* electric organ.

After SNAP-25 proteolysis by BoNT/A, transition of the ternary SNARE complex in a SDS-resistant state still occurs (Hayashi *et al.*, 1994) (Figure 19.5); therefore, BoNT/A (or BoNT/C) is unlikely to abolish irreversibly the fusion process *per se*. The C-terminal domain of SNAP-25, which contributes one helix to the four-helix bundle, has been implicated in the final step of $Ca^{2+}$-dependent exocytosis (Chen *et al.*, 1999). Truncation of SNAP-25 by BoNT/A or BoNT/E alters both SNAP-25 and $Ca^{2+}$ binding to synaptotagmin (Gerona *et al.*, 2000; Tucker *et al.*, 2004). Thus, a likely model for the blocking action of BoNT/A (and partially for BoNT/C) is that removal of the nine C-terminal amino acids of SNAP-25 deeply disrupts the coupling between $Ca^{2+}$ sensing and the final step in exocytosis (Figure 19.5, top panel). Overall, this is consistent with the observation that any treatment aimed at increasing $Ca^{2+}$-influx forces the blocked step and rescues synaptic transmission at BoNT/A-treated synapses (Table 19.4), albeit this latter action may also refer to interactions between the SNAREs and other $Ca^{2+}$-dependent partners of the secretion machinery (Quetglas *et al.*, 2000; Zhang *et al.*, 2002). Another possibility has to be considered: SNAP-25 cleaved by BoNT/A (or BoNT/C) maintains its association with syntaxin (Bajohrs *et al.*, 2004), possibly slowing the docking (i.e., formation of the heterotrimeric fusion complex comprised of SNAP-25, syntaxin, and VAMP) or favoring a complex composed of a second syntaxin molecule leading to a stable four-helix complex obstructing the binding site for VAMP (Fasshauer and Margittai, 2004). Therefore, truncated SNAP-25 can behave as a dominant negative mutant upon the exocytotic process. This additional consequence of SNAP-25 cleavage is fully consistent with: (i) blocking release due to both functional elimination of SNAP-25 and accumulating cleavage product that competitively inhibits exocytosis (Keller and Neale, 2001); and (ii) BoNT/A slows down a secretory step before fusion *per se*, and before the step affected by BoNT/E (Xu *et al.*, 1998).

The mechanisms by which BoNT/E blocks secretion are quite different from those of BoNT/A. Truncation of SNAP-25 by BoNT/E destabilizes the four-helix bundle of the SNARE complex. This is not enough to explain the inhibitory action of BoNT/E because discontinuous SNAP-25 mediates fusion (Chen *et al.*, 1999; Chen *et al.*, 2001). Two other factors have to be taken into account. First, SNAP-25 truncated by BoNT/E is not retained by syntaxin (Bajohrs *et al.*, 2004) and is rapidly eliminated. Second, inhibition of exocytosis by BoNT/E can be rescued by supplementing the C-terminal portion of SNAP-25 removed by the toxin (Chen *et al.*, 1999; Chen *et al.*, 2001; Schuette *et al.*, 2004). Therefore, this means that the C-terminal 26mer released upon proteolysis of SNAP-25 by BoNT/E is lacking at the release sites. In fact, it is likely to diffuse far away from the active zone because of its diffusional sinking in the very large axon volume. This also suggests that although expression of this fragment inhibits exocytosis, possibly because it destabilizes or prevents formation of the four-helical fusogenic bundle (Ferrer-Montiel *et al.*, 1998), it is unlikely that the C-terminal 26mer contributes significantly to the BoNT/E-induced inhibitory action. Thus, blockade of exocytosis by BoNT/E is only due to cleavage of SNAP-25, not the production of competitive antagonists of SNARE complex formation, as discussed above for BoNT/A. This also agrees with the findings that treatments enhancing the release probability have a low efficacy for restoring muscle contraction at BoNT/E-poisoned motor-nerve terminals (Table 19.4). Thus, blockade of exocytosis by BoNT/E is only due to elimination of functional SNAP-25 and not due to production of competitive antagonists of SNARE complex formation, as discussed above for BoNT/A. This also agrees with

findings that treatments for enhancing the release probability are minimally efficacious for restoring muscle contraction at BoNT-E-poisoned motor-nerve terminals (Table 19.4).

*Blockade induced by VAMP cleavage*

The causal relationship between proteolysis of VAMP by TeNT, BoNT/B, D, or F, and preventing exocytosis was first deduced from observations that: (i) peptides encompassing the VAMP cleavage site for TeNT, or BoNT/B, can compete with VAMP and diminish the inhibitory action of these neurotoxins; and (ii) presynaptic injection of VAMP-specific antibodies into *Aplysia* cholinergic neurons prevents specifically the blocking action of TeNT or BoNT/B, but not that of BoNT/A, which cleaves SNAP-25. Furthermore, transfection of insulin-secreting cells with mutated VAMP not cleaved by TeNT allows partial restoration of secretory properties (Humeau *et al.*, 2000; Schiavo *et al.*, 2000). In addition, immunocytochemical studies with rat hippocampus (Capogna *et al.*, 1997), as well as frog (Raciborska *et al.*, 1998), crayfish (Hua and Scheller, 2001), and mouse (Kalandakanond and Coffield, 2001b; Whelchel *et al.*, 2004) NMJs reveal that blocked neurotransmitter release correlates well with diminished VAMP immunoreactivity. This correlation is far less clear in catecholaminergic synaptosomes in which almost complete inhibition of evoked release was observed with partially proteolyzed VAMP. The fact that BoNT cleaves VAMP at distinct sites has pertinent physiological correlates. Thus, at mouse motor terminals, both VAMP-1 and -2 can be cleaved by TeNT, or BoNT/B, and there is a significant decrease in spontaneous quantal release (Table 19.1). In contrast, rat VAMP-2 but not VAMP-1 are cleaved by TeNT and BoNT/B (Table 19.5) with a concomitant small decrease in spontaneous quantal release at the NMJ treated with TeNT, despite a clear decrease in rat hippocampal synapses (Capogna *et al.*, 1997; Humeau *et al.*, 2000). BoNT/D and BoNT/F cleave both VAMP-1 and -2 rat isoforms, as well as strongly diminish spontaneous quantal release at rat motor nerve endings. At the crayfish motor synapses, BoNT/D significantly reduces the frequency of spontaneous quantal events unlike BoNT/B- and TeNT-L chains, which share the same cleavage site (Hua *et al.*, 1998).

How does VAMP cleavage inhibit secretion? *In vitro* studies indicate that cleavage of VAMP-2 by TeNT, BoNT/B, D, F, or G does not completely prevent assembly of VAMP-2, SNAP-25, and syntaxin into ternary SNARE complexes, but noticeably fewer complexes are formed following TeNT and BoNT/B treatment (Yersin *et al.*, 2003). Two possible scenarios can account for blocked exocytosis induced by these toxins. First, when VAMP is cleaved by TeNT, BoNT/B, or BoNT/G, the VAMP portion (~20 amino acids) remaining in the synaptic vesicle membrane does not contain interaction sites for the other SNAREs. Therefore, the synaptic vesicle membrane is no longer linked to a SNARE complex, and fusion with the plasma membrane cannot occur. Second, when VAMP is cleaved by BoNT/D or F, the C-terminal fragment remaining in the vesicle membrane is long enough to anchor the synaptic vesicle to the SNARE complex, but fusion cannot occur because the thermally stable, four-helix bundle cannot be formed. Hence, despite the appearance of docked synaptic vesicles, treatments that increase neurotransmitter release remain unsuccessful in reversing toxin action (Table 19.4). An additional component during the blockade is a VAMP cytoplasmic fragment released upon proteolysis by TeNT or BoNT/B (Cornille *et al.*, 1995; Tucker *et al.*, 2004). Possibly, this fragment competes with intact VAMP and prevents pairing of the SNAREs during complex formation and vesicle docking.

How can one explain the asynchronous release observed after treatment with TeNT, BoNT/B, and BoNT/D? Several recent studies suggest that fusion of the synaptic vesicle with the plasma membrane requires several (possibly three?) SNARE complexes (Poirier *et al.*, 1998; Hua and Scheller, 2001). Our speculation is that the few desynchronized exocytotic events, which occur when the release probability is increased at TeNT-, BoNT/B-, BoNT/D-treated NMJs, might represent a small fraction of synaptic vesicles that are docked to the release site by both functional and disabled SNARE complexes. In this regard, some of the docked vesicles observed after VAMP-cleavage might correspond to synaptic vesicles anchored to the plasma membrane by an incomplete set of SNARE complexes, as well as to vesicles tethered to the plasma membrane by other proteins like Rab3 bound to Rim, and synaptotagmin bound to the plasma membrane or $Ca^{2+}$-channels.

*Blockade induced by syntaxin cleavage*

BoNT/C cleaves both syntaxin-1 and SNAP-25; therefore, determining which of these two targets is cleaved during BoNT/C-induced blockade is an important issue for understanding BoNT/C mechanisms of action. *In vitro* cleavage of SNAP-25 by BoNT/C occurs with low efficiency (~1000-fold difference) versus cleavage by BoNT/A or E (Humeau *et al.*, 2000; Schiavo *et al.*, 2000). Immunocytochemical studies with frog and mammalian NMJs show that immunoreactivity of the SNAP-25 C-terminus (removed following BoNT/A, C, or E action) remains nearly intact when syntaxin immunoreactivity is

greatly decreased (Raciborska *et al*., 1998; Kalandakanond and Coffield, 2001b). However, in cultured hippocampal slices or spinal neurons, BoNT/C is very efficient at removing nearly all SNAP-25 immunoreactivity (Capogna *et al*., 1997). A clear-cut demonstration of the causal relationship between syntaxin cleavage and blockade of neurotransmitter exocytosis has been obtained with the giant squid synapse. BoNT/C cleavage of squid syntaxin-1, but not squid SNAP-25 (O'Connor *et al*., 1997), completely inhibits transmitter release. Additionally, the secretory blockade in chromaffin cells by BoNT/C is very distinct from that produced by BoNT/A (Xu *et al*., 1998). Furthermore, the action of BoNT/C is very similar to the blockade of both evoked and spontaneous quantal transmitter release that characterizes *Drosophila* mutants lacking syntaxin. Thus, depending on the cell type, the secretory blockade is likely due to syntaxin and/or SNAP-25 cleavage.

A possible molecular model for BoNT/C-induced inhibition of exocytosis involves SNARE complex formation that despite a tight, SDS-resistant, four-helix bundle, facilitates loose docking, but not fusion, of the synaptic vesicle to the plasma membrane. The vesicles remain tethered to the plasma membrane via SNAP-25 and other proteins (Rab3/Rim, synatotagmin, etc.). This is consistent with altered scaffold ribs at the release site of NMJs poisoned with BoNT/C, but not BoNT/A (Stanley *et al*., 2003). The blocked exocytosis evident after BoNT/C cleavage of SNAP-25 is similar to that produced by BoNT/A (see above).

*Common consequences of SNARE cleavage on exocytosis*

Despite the scenario that blocking actions by various BoNTs and TeNT differ at the molecular level, they all share several commonalities. Indeed, in all cases following toxin action, the SNARE complex is no longer fusogenic. Thus, synaptic vesicles remaining docked at the fusion site with slightly increased numbers suggest that when tethered to the plasma membrane or docked in an unproductive complex, not only exocytosis but also undocking cannot proceed. Therefore, it is likely that synaptic vesicles docked with unproductive complexes can irreversibly plug the fusion sites that would normally accommodate intact vesicles. Obviously, this should amplify toxin effects and may also explain why there is some discrepancy between the amount of SNARE cleaved and the extent of blocked transmitter release (Kalandakanond and Coffield, 2001a).

### *Other cell consequences of SNARE cleavage*

*Alteration of the vesicle cycle beyond fusion*

Each step of the synaptic vesicle cycle involving SNAREs may be affected by toxin. For example, VAMP cleavage abolishes its interaction with the adaptator protein AP3, and affects synaptic vesicle recycling via early endosomes (Salem *et al*., 1998). Although interaction of synaptophysin-1 with VAMP-1 is controversial, a report that the cytosolic cleavage product of VAMP-2, but not VAMP-1, released upon TeNT or BoNT/B activity blocks neurotransmitter release (Cornille *et al*., 1995) may refer not to an alteration of the SNARE complex function, but to a disturbance in the synaptophysin-1/VAMP-2 interaction.

*Alteration of $Ca^{2+}$- and $K^{+}$-channel functions*

Different voltage-gated $Ca^{2+}$-channels (N-, P/Q-, R-, and L-types) are involved in neurotransmitter and hormone release by neurons, as well as excitable neuroendocrine and endocrine cells. In addition, store-mediated $Ca^{2+}$ entry allows $Ca^{2+}$ influx in nonexcitable cells (Augustine, 2001; Rettig and Neher, 2002; Petersen, 2003). Syntaxin promotes inactivation of N-, P/Q-, R-, or L-type $Ca^{2+}$ channels, and this inactivation is removed when proteins of the fusion complex (i.e., SNAP-25, VAMP, synaptotagmin, etc.) bind to syntaxin and/or $Ca^{2+}$ channels (Wiser *et al*., 1999; Degtiar *et al*., 2000; Stanley *et al*., 2003; Cohen and Atlas, 2004). In this line, SNAP-25 controls $Ca^{2+}$ responsiveness to depolarization (Verderio *et al*., 2004). These findings support the hypothesis that t-SNAREs not only play a key role in exocytosis, but also control $Ca^{2+}$ dynamics. Possibly, docking of synaptic vesicles to the fusion site (i.e., formation time of the ternary SNARE complex) reactivates $Ca^{2+}$ channels, thereby making possible $Ca^{2+}$ influx only at the sites occupied with readily releasable synaptic vesicles (Atlas, 2001).

The syntaxin domain (synprint) interacting with the $\alpha$-subunit from a $Ca^{2+}$ channel is very close to the site cleaved by BoNT/C. The question is whether cleavage of syntaxin by BoNT/C, or cleavage of other SNAREs by other BoNTs or TeNT, might inhibit $Ca^{2+}$ influx and thereby contribute to the toxin-induced blockage of exocytosis. Cleavage of syntaxin by BoNT/C abolishes the regulation of $Ca^{2+}$ channels by heterotrimeric G-protein (Stanley and Mirotznik, 1997). However, despite a complete block of neurotransmitter release by BoNT/C in isolated mammalian nerve terminals, no clear alteration of evoked fast $Ca^{2+}$ entry was detected, while slow $Ca^{2+}$ influx was potentiated (Bergsman and Tsien, 2000). In contrast, $Ca^{2+}$ entry was significantly decreased by BoNT/C in *Torpedo* preparations (Aleu *et al*., 2002). Cleavage of VAMP or SNAP-25 with the other BoNTs failed to produce a significant change in $Ca^{2+}$ influx (Bergsman and Tsien, 2000; Aleu *et al*., 2002), thus confirming previous results obtained at motor nerve terminals of the NMJs (Molgo *et al*., 1989). Overall, these findings suggest that alterations in $Ca^{2+}$

influx at exocytotic sites are unlikely to significantly inhibit neurotransmitter release from neurons.

However, this is not the case for secretion from other cell types. Indeed, SNAP-25 modulates L-type channels (Ji *et al*., 2002) and entry of $Ca^{2+}$ mediated by store-operated channels in pancreatic acini that secrete amylase, or human platelets. In these cells, $Ca^{2+}$ influx is strongly depressed after SNAP-25 cleavage, possibly blocking $Ca^{2+}$-dependent exocytosis by BoNT/A (Rosado *et al*., 2005). Because of a very high concentration of toxin (~100 nM), this effect is unlikely to have any pathophysiological relevance for botulism.

With the same thoughts as presented above, physical as well as functional interactions between syntaxin-1A and voltage-gated, $K^+$-channels (Kv1.1 type) of the brain have been discovered recently (Fili *et al*., 2001). Activity of the Kv2.1 channel, a prevalent delayed rectifier channel in endocrine and neuroendocrine cells, is also strongly modulated by syntaxin and SNAP-25 (Michaelevski *et al*., 2003; Tsuk *et al*., 2004). Regulation of $K^+$ channels by SNARE proteins may play a role in membrane excitability and exocytosis. This raises the question of whether exocytosis may be altered by BoNTs via changes in activation of Kv2.1 currents.

## Putative, non-proteolytic molecular actions of BoNTs and TeNT

Several controversial observations support the idea that additional mechanisms may contribute to the neuroinhibitory action of BoNTs and TeNT. Indeed, intracellular application of TeNT mutants devoid of proteolytic activity *in vitro* into intact neurons induces substantial blockade of neurotransmitter release (Ashton *et al*., 1995). Tissue transglutaminase type II is reportedly activated (non-proteolytically) by TeNT, affecting neurotransmitter release via the synapsins (Humeau *et al*., 2000). Another unexpected effect of TeNT is modification of the actin cytoskeleton, which involves inhibited rearrangements of subcortical microfilaments that accompany secretion in chromaffin cells (Marxen and Bigalke, 1991). This is consistent with TeNT-induced alteration of the actin cytoskeleton when TeNT light chain is expressed in murine Sertoli cells (Eisel *et al*., 1993). TeNT also affects the depolarization-stimulated phosphorylation and redistribution of synapsin I (Presek *et al*., 1992). However, this may refer both to changes in the actin cytoskeleton organization and activation of tissue transglutaminase type II by TeNT. Furthermore, DasGupta and Tepp (1993) published the *in vitro* observation that BoNT/E light chain cleaves actin at every potential site (n=11) involving Arg or Lys residues in the *P*′1 position, exactly as that found in SNAP-25. In line with the mentioned cytoskeletal alterations, and consistent with the well documented implication of small GTPases like Rho in the dynamics and organization of actin-based cytoskeletons (Hall, 1998), BoNT/A reportedly targets RhoB to the proteasome, causing both blockade of exocytosis and actin cytoskeleton disorganization (Ishida *et al*., 2004). This possibly reflects a cross talk between remodeling of the actin cytoskeleton and SNARE-/Rho-GTPase-dependent mechanisms of exocytosis, as recently illustrated for Cdc42 and VAMP-2 during insulin secretion (Nevins and Thurmond, 2005).

The intracellular action of TeNT is also associated with changes in synaptic phosphoinositide levels and/or protein kinase C activity (Humeau *et al*., 2000). This action of TeNT involves the binding domain, because the C-terminal fragment of TeNT heavy chain activates protein kinase signaling pathways in cultured cortical neurons (Gil *et al*., 2003; Chaib-Oukadour *et al*., 2004). Possibly, the unexpected inhibition of $Na^+$-dependent uptake of 5-hydroxy trytamine (5HT) induced by the C-terminal half of TeNT heavy chain (Inserte *et al*., 1999; Najib *et al*., 1999) is linked to these pathways. This suggests that the acceptor for TeNT is coupled to a tyrosine kinase, receptor-dependent pathway.

## Long-term effects of BoNTs

### *BoNTs trigger sprouting of motor neurons and differentiation of new endplates*

A local injection of BoNT into adult skeletal muscles of animals and humans blocks quantal ACh release, resulting in flaccid muscle paralysis namely restricted to the injection site. Although a marked atrophy of skeletal muscle occurs during paralysis, there is no evident damage of motor nerve terminals. The duration of neuromuscular blockade (Table 19.8) depends upon: (i) BoNT serotype; (ii) dose of BoNT; and (iii) animal species. In general, higher doses are needed to paralyze amphibian, versus mammalian, skeletal muscles (Poulain *et al*., 2000). An attractive feature of using BoNTs is that the neuromuscular blockade in any animal species is not permanent, as recovery of neuromuscular transmission returns within a few weeks to months.

In mammals, muscle paralysis following a single injection of BoNT/A elicits within 3–5 days a nerve outgrowth or sprouting that occurs along intramuscular axons at the nodes of Ranvier (nodal sprouting) and at motor nerve terminals of the NMJs (terminal sprouting). Nodal sprouts are more metabolically costly to the motoneuron than are terminal sprouts, which predominate in mature muscles treated with BoNT/A. Nerve terminal sprouting is also produced by other agents

**TABLE 19.8** Duration of skeletal muscle paralysis induced by various BoNTs in humans and rodents

| | BoNT/A | BoNT/B | BoNT/C | BoNT/E | BoNT/F |
|---|---|---|---|---|---|
| Human* | 4–6 months | 2–3 months | 4–5 months | 4–6 weeks | 2 months |
| Rodents** | 21–30 days | 7–10 days | 21–30 days | 3–5 days | 7–10 days |

*Human values were obtained in different skeletal muscles after a single BoNT local injection.
**Data were obtained by either monitoring *in vivo* the recovery of nerve-evoked muscle contraction via the digit abduction score assay, or *ex vivo* via intracellular electrophysiological recordings.

that render the muscle inactive via prolonged blockade of either nerve conduction or muscle nAChRs. Interestingly, restoration of muscle activity by direct stimulation prevents nerve sprouting induced by BoNT/A (Brown *et al*., 1981).

Sprouting is greater in younger animals, generally increases with time after BoNT injection, and depends upon the contractile properties of the muscle as defined by its innervation pattern. Thus, sprouting is more prominent in slow (e. g., *soleus*), than in fast (e. g., *extensor digitorum longus*), contracting muscles. In addition, sprouting is more abundant in muscles innervated by short axons (Meunier *et al*., 2002). Nerve terminal sprouts evoked by BoNT/A in mouse *levator auris longus* muscle appear as thin, unmyelinated filaments usually oriented parallel to the longitudinal axis of the muscle extending beyond the original nerve terminal (Angaut-Petit *et al*., 1990; Molgo *et al*., 1990; de Paiva *et al*., 1999). After a BoNT/A injection, the sprouts usually increase in length as well as complexity for about 40–50 days and continue to grow for 30–40 days, despite the recovery of nerve-evoked muscle twitches (Juzans *et al*., 1996a), as confirmed by time-lapse imaging of the same mouse NMJ (de Paiva *et al*., 1999). The persistence of nerve terminal sprouts has also been reported in the human orbicularis muscle after repeated injections of BoNT/A (Holds *et al*., 1990). Nerve terminal sprouting was also detected in mouse *levator auris longus* muscle injected with BoNT/D (Comella *et al*., 1993) and BoNT/C (Molgo *et al*., unpublished), as well as in mouse sternomastoid muscle injected with BoNT/F (Meunier *et al*., 2003). Interestingly, no sprouts were visible in the mouse sternomastoid muscle following BoNT/E injection (Meunier *et al*., 2003), which is probably due to short-lived muscle inactivity since recovery from paralysis starts within 2–3 days after neurotoxin administration.

In BoNT/A-paralyzed muscles, the glial component of the NMJ (i. e., the perisynaptic Schwann cell also known as the *terminal Schwann cell* that covers nerve terminal arborizations) plays an important role by inducing and guiding sprouts along muscle fibers. Schwann cells extend processes into every nerve terminal sprout. In some cases, these processes appear even longer than the sprouts growing beside them (Son and Thompson, 1995). The perisynaptic Schwann cells sense synaptic activity at the NMJ and regulate expression of certain genes. Thus, blockade of transmitter relaese from motor nerve terminals is known to evoke gene expression for the glial fibrillary acidic protein (GFAP) (Georgiou *et al*., 1994). This protein may be important in extending perisynaptic Schwann cell processes. Schwann cells produce various neurotropic factors and also synthesize cell adhesion molecules, such as N-CAM, Ng-CAM/L1, N-cadherin, and L2/HNK-1, which form basement membranes containing many extracellular matrix proteins like laminin, fibronectin, and tenascin. The CGRP neuropeptide reportedly stimulates Schwann cell proliferation by increasing cyclic adenosine monophosphate (cAMP) levels (see below) that regulate mitogenic factors (Cheng *et al*., 1995). Apart from a presynaptic role in guiding sprouts triggered by BoNTs, perisynaptic Schwann cells may also play a postsynaptic role by expressing active agrin isoforms that enhance aggregation of nAChRs on muscles (Yang *et al*., 2001).

Electrophysiological focal recordings in the original nerve terminal and along newly formed terminal sprouts provide evidence that sprouts are endowed with voltage-dependent $Na^+$ channels, allowing an active propagation of action potentials throughout their length. Additionally, the presence of voltage-dependent $Ca^{2+}$ channels and $Ca^{2+}$-dependent, $K^+$ channels in the sprout membrane has been reported (Angaut-Petit *et al*., 1990). At mature NMJs, P/Q type voltage-dependent $Ca^{2+}$ channels are coupled to the evoked release process (Van der Kloot and Molgó, 1994). However, blockers of L- and N-type $Ca^{2+}$ channels can reduce nerve-evoked neurotransmitter release during the first four weeks following an injection of BoNT/A (Santafe *et al*., 2000). This time period is characterized by an abundance of nerve terminal sprouts. Interestingly, a transient involvement of both L- and N-type $Ca^{2+}$ channels coupled to neurotransmitter release has also been reported in motor nerve terminals of new-born mammals (Rosato Siri and Uchitel, 1999).

Thus, it is likely that the transient expression of different $Ca^{2+}$ channels may be necessary for activating regulatory pathways during synaptogenesis induced by BoNTs. Thereafter, and upon functional recovery of neuromuscular transmission in BoNT/A-treated muscles, only P/Q-type $Ca^{2+}$ channel blockers affect evoked neurotransmitter release.

During the growth and maturation of BoNT/A-induced sprouts, there is a sequential appearance of intrinsic cytoskeletal axonal components like neurofilaments and tubulin that can be visualized using immunocytochemical staining (Alderson *et al*., 1989). In addition, after about two weeks post-injection of BoNTs, synaptic proteins like synaptophysin, synaptotagmin-II (Juzans *et al*., 1996a; Juzans *et al*., 1996b), VAMP, and SNAP-25 (de Paiva *et al*., 1999; Meunier *et al*., 2003) accumulate in sprouts at synaptic contact points with muscle fibers and growth cones. In addition, clustering of postsynaptic nicotinic ACh receptors (nAChRs) with the growing sprouts can also be visualized with rhodamine-labeled α-bungarotoxin.

During BoNT/A-induced synaptic remodeling, there is a large increase in immunoreactivity for CGRP (Hassan *et al*., 1994; Sala *et al*., 1995; Meunier *et al*., 2002). CGRP is synthesized by motoneurons and transported to motor nerve terminals where it is stored in large, dense-core vesicles that are released upon stimulation (Van der Kloot and Molgó, 1994). An increase in CGRP observed during nerve terminal outgrowth can only partially account for the accumulation of vesicles containing CGRP in the original nerve terminus and sprouts (Meunier *et al*., 2002). Such remarkable CGRP accumulation in BoNT/A-treated nerve terminals results probably not only from inhibition of CGRP release by the neurotoxin, as recently shown in primary cultures of rat trigeminal neurons (Durham *et al*., 2004), but also from upregulated synthesis in motoneuron's somata (Sala *et al*., 1995; Tarabal *et al*., 1996).

*In vivo* (Buffelli *et al*., 2001) and tissue-culture studies (New and Mudge, 1986; Fontaine *et al*., 1987) have consistently shown that CGRP increases nAChR mRNA and protein expression, with a subsequent increase in nAChR molecules on muscle cell fibers. This appears mediated by increased levels of intracellular cAMP with a concomitant activation of protein kinase A (PKA). Therefore, it can be hypothesized that the release of CGRP from nerve terminal sprouts may provide a localized signal to stimulate nAChR gene expression by subsynaptic nuclei, thus resulting in a localized nAChR synthesis along the sprouts. However, given the redundant factors involved in nAChR accumulation, further experiments are needed before finally concluding that CGRP is an endogenous factor by which motoneurons regulate expression of junctional nAChRs along the sprouts. Interestingly, recent studies indicate that CGRP also regulates and maintains acetylcholinesterase expression during the development of NMJs (Rossi *et al*., 2003). Available evidence indicates that nerve terminal sprouts and the muscle fiber possess the molecular machinery for ACh release and its detection, supporting the view that terminal sprouts play a role in recovering neuromuscular transmission following BoNT/A treatment. Figure 19.6 shows a typical example of the electrophysiological characteristics during blockade of neuromuscular transmission, and its recovery, following the action of BoNT/A.

Progression of events leading to the maturation of newly-formed sprouts, development of the first nerve-muscle contacts, and differentiation of new endplates after BoNT exposure is a remarkable demonstration of synaptic plasticity in mature NMJs. Throughout this period, important communications occur that involve multiple stages and signaling molecules between synaptic partners like the perisynaptic Schwann cells, nerve terminal, nodal sprouts, and muscle fibers. Paralysis results in up-regulation of cell surface and basal lamina components that promote axonal

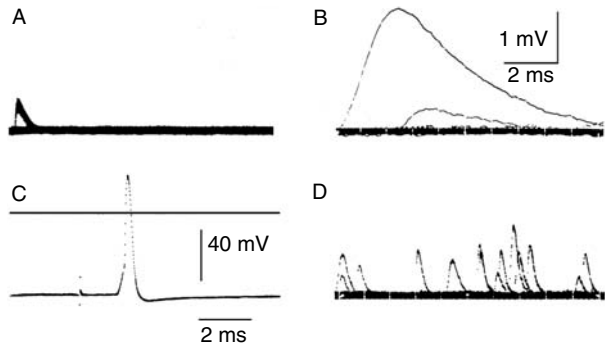



**FIGURE 19.6** Electrophysiological recordings obtained from junctions in mouse muscles injected with BoNT/A at 3 (A), 5 (B) and 30 days (C, D) post-toxin injection. Panel A. Oscilloscope superimposed tracings of endplate potentials evoked by nerve stimulation at 0.5 Hz. Note that only 15% of nerve impulses give rise to synaptic responses, while 85% of nerve impulses failed to evoke transmitter release. Panel B. Spontaneous endplate potential recorded in another junction during prominent sprouting of motor nerve endings. Note, the same time-scale as in A. The ACh giving rise to these spontaneous events does not contribute to evoked transmitter release. Panel C. Nerve-evoked muscle action potential triggered by synaptic activity. The upper line denotes the 0 mV membrane level. Panel D. Superimposed tracings of spontaneous miniature-endplate potentials recorded from another junction of the same muscle as in panel C, in which neuromuscular transmission has recovered. All recordings were performed at 37°C in standard Krebs-Ringer solution.

growth (Sanes and Lichtman, 1999), and some of these molecular components could possibly guide sprouts along muscle fibers.

Direct electrical stimulation of muscle previously paralyzed by Botulinum complex/A reportedly prevents sprouting (Brown *et al*., 1981), which likely involves muscle-derived signaling factors in sprout generation. Expression of insulin-like growth factors (IGF) is evidently up-regulated in BoNT/A-paralyzed muscle, and IGF-binding proteins delivered locally to paralyzed muscles prevent nerve sprouting (Caroni *et al*., 1994). An increase in muscle plasminogen activator has also been described during paralysis induced by BoNT/A (Tian *et al*., 1995), and it is expected that this protease may disintegrate some components of the junctional basal lamina that play a role in neuromuscular formation and plasticity.

***Changes induced by BoNTs in skeletal muscle fibers***

One striking feature of skeletal muscles paralyzed with BoNT/A is that they undergo a progressive atrophy, which begins during the first week after toxin injection and progresses throughout the following weeks. A more rapid progression of atrophy following BoNT/A injection has been reported in "slow," versus "fast," contracting muscles. These changes are characterized by a noticeable decrease in muscle weight following diminished fiber diameter, as compared to controls. The atrophy of skeletal muscle is usually rather heterogeneous between fibers of a given muscle, probably because some unpoisoned fibers maintain their normal diameter. The muscle atrophy induced by BoNTs in animal models and humans is largely reversible, even after repeated BoNT injections.

The fact that NMJs remain intact in BoNT-paralyzed muscle, since no degenerative changes are detected among its components, does not preclude the presence of some denervation-like alterations. Thus, muscle fibers treated with BoNT/A exhibit a small decrease in resting membrane potential, develop spontaneous fibrillation and action potentials (directly elicited by electrical stimulation), which become partially resistant to tetrodotoxin, which normally blocks voltage-sensitive $Na^+$ channels (Thesleff, 1989; Thesleff *et al*., 1990). Insensitivity towards tetrodotoxin results from an overexpression of mRNA encoding tetrodotoxin-insensitive, voltage-dependent $Na^+$ channels ($Na_V1.5$/SkM2) (Yang *et al*., 2001). In human adult, BoNT/A-treated skeletal muscles, such insensitivity declines when functional recovery of neuromuscular transmission occurs. In addition to these changes, muscles treated with BoNT/A become susceptible to innervation by foreign nerves. During development, BoNT/A-blockade delays and impedes the retraction of polyneuronal innervation in muscle and prevents motoneuron cell death (Thesleff *et al*., 1990). In addition, BoNT/A-paralyzed muscles express a high endocytotic activity limited to the endplate region (Thesleff *et al*., 1990). When taken altogether, these observations support the view that paralyzed muscles secrete factors essential for growth and survival of motoneurons.

Every adult skeletal muscle fiber contains an important population of nuclei, a few of which cluster into the endplate region. These synaptic nuclei are larger and more round than extrasynaptic nuclei, and they are transcriptionally specialized, since they express genes that encode different synaptic proteins, which include nAChR subunits at levels far higher than those of extrasynaptic nuclei (Burden, 1993). After treatment with BoNT/A, atrophic muscle fibers contain a large number of synaptic nuclei located not only in the original endplate area, but also in the vicinity of nerve terminal sprouts. Concomitant with these changes, spatial distribution and levels of the different subunit-specific mRNAs encoding nAChRs also vary. Consequently, mRNA levels for the alpha-subunit of the nAChR increase (Lipsky *et al*., 1989), and changes in the levels as well as distribution of gamma- and epsilon-subunit specific mRNAs correlate well with fetal and adult nAChR channel subtypes appearing along muscle fibers (Witzemann *et al*., 1991; Koltgen *et al*., 1994). In BoNT/A-paralyzed muscle fibers, the density of nAChRs increases dramatically, as determined by specific binding with radiolabeled [$^{125}$I]-α-bungarotoxin (Bambrick and Gordon, 1987), or by fluorescence staining with rhodamine-labeled α-bungarotoxin. This explains the ACh supersensitivity observed a few weeks after poisoning in BoNT-poisoned muscles (Tonge, 1974; Thesleff *et al*., 1990). The increase in $^{125}$I-α-bungarotoxin binding sites induced by BoNT/A occurs to a lesser extent in neonatal, than in adult, rat skeletal muscles (Bambrick and Gordon, 1992). Detection of nAChRs in muscles injected with BoNTs reveals clear reorganization of the postsynaptic elements within NMJs, with distinguishable patches of α-bungarotoxin staining abutting the sprouts. Paralysis by BoNT/A is also associated with changes in the molecular pattern of acetylcholinesterases anchored to the basal lamina of skeletal muscle.

In conclusion, after axonal sprouting and reformation of functional NMJs, the muscle eventually regains its normal size and both acetylcholinesterase and nAChR reconcentrate at the NMJ. Sprouts largely degenerate and the endplate eventually regains its normal morphology and function.

### *Duration of BoNT actions*

One of the important aspects of BoNT action is the duration of muscle paralysis. As shown in Table 19.8, the average duration of muscle paralysis induced by BoNT/A in humans and rodents exceeds that resulting from exposure to other BoNT serotypes, with the exception of BoNT/C, which has a slightly less duration of action than BoNT/A (Eleopra *et al*., 1998; Meunier *et al*., 2003). Thus, according to their duration of action, BoNTs can be classified into three groups: (i) long-lasting (BoNT/A and BoNT/C); (ii) intermediate (BoNT/B and BoNT/F); and (iii) short-lasting (BoNT/E). It is still unclear why the functional recovery time in rodents is shorter than in humans after BoNT exposure.

Proteolytic activity of BoNT/A and C, with the appearance of cleavage product in cultured neurons or chromaffin cells, persists beyond 80 days, whereas activity of the other BoNTs like BoNT/E is much shorter (<1 day) (Keller *et al*., 1999; O'Sullivan *et al*., 1999; Foran *et al*., 2003; Whelchel *et al*., 2004). Long-term persistence of BoNT/A, and possibly BoNT/C molecules inside nerve terminals, may provide an explanation for different durations of inhibition for these two toxins (O'Sullivan *et al*., 1999; Foran *et al*., 2003). BoNT/A, but not BoNT/E, L-chains possess a sorting signal (di-leucine motif), which may facilitate interaction with adaptator proteins and redirection to the active zones (Fernandez-Salas *et al*., 2004). However, the timescale for BoNT/E-induced paralysis was surprisingly shorter than that for BoNT/A (Eleopra *et al*., 1998; Meunier *et al*., 2003). It has been hypothesized that truncated SNAP-25 lacking a C terminal 9mer, released by BoNT/A proteolysis, has a longer life span than SNAP-25 with a 26mer deletion induced by BoNT/E (Keller *et al*., 1999; Humeau *et al*., 2000; Meunier *et al*., 2003). Recent findings support strongly this possibility as evidenced by SNAP-25 truncation by BoNT/A, but not BoNT/E, and subsequent interactions with syntaxin (Bajohrs *et al*., 2004). The syntaxin/SNAP-25 complexes formed with BoNT/A-truncated SNAP-25 induce long-term retention within the active zone (Bajohrs *et al*., 2004). Since this SNAP-25 fragment is not fusogenic, long-term impeding of synaptic vesicle fusion becomes evident. Possibly, membrane retention prevents degradation of SNAP-25 by intracellular proteases. In contrast, the SNAP-25 fragment generated by BoNT/E cannot bind to syntaxin. SNAP-25 produced by BoNT/A also acts as substrate for BoNT/E (Lawrence *et al*., 1997). Therefore, the long life span of the unproductive complex comprising syntaxin and SNAP-25 truncated by BoNT/A is shortened by further cleavage with BoNT/E, thus explaining how BoNT/E shortens paralysis induced by BoNT/A.

## OTHER TOXINS WITH DISTINCT NEUROTOXIC ACTIVITIES

### *Clostridium perfringens* epsilon toxin

Epsilon toxin acts on different cell types, including those of neuronal origin. It is not a strict neurotoxin as defined previously for the BoNTs and TeNT, but epsilon toxin is involved in neurological symptoms. Epsilon toxin represents the major virulence factor of *C. perfringens* types B and D, which are the etiological agents of fatal enterotoxemia in sheep as well as goats, and more rarely of cattle (see Chapter 35 of this volume). Overgrowth of *C. perfringens* in the intestine of susceptible animals, generally a consequence of overeating high starch/sugar-containing foods, results in large amounts of epsilon toxin. The toxin is absorbed through the intestinal mucosa and spreads into different organs by the circulatory system, ultimately causing hypertension, vascular permeability, lung edema, and kidney necrosis (pulpy kidney disease in lambs). The disease is rapidly fatal with neurological disorders that include excitation symptoms.

Major pathological changes are observed in the brain following epsilon intoxication, which include: congestion and edema of the meninges; perivascular and intercellular edema; as well as necrotic foci of the nervous tissue. Epsilon toxin passes through the blood-brain barrier and accumulates specifically in the brain (Nagahama and Sakurai, 1991; Nagahama and Sakurai, 1992). The toxin damages endothelial cells of the blood-brain barrier by altering the endothelial barrier antigen localized to luminal endothelial cell membranes, thus causing leakage of endogenous albumin (Zhu *et al*., 2001). The specific receptor for epsilon toxin on neuronal and endothelial cells is not yet known, although a membrane sialoglycoprotein seemingly mediates toxin binding (Buxton, 1978; Nagahama and Sakurai, 1992). In the brain, epsilon toxin increases vascular permeability, leading to a rapid, severe, and diffuse edema that exerts a direct cytotoxic effect upon neurons. Neuronal damage is characterized by progressive cytoplasmic vacuolization plus necrosis, and in some cells there is hyperchromatosis and nuclear pyknosis (Finnie *et al*., 1999).

The neurological disorders linked to epsilon toxin, which likely result from an excessive release of glutamate, include retraction of the head, opisthotonus, convulsions, agonal struggling, and hazardous roaming. Indeed, epsilon toxin injected intravenously into mice preferentially excitates hippocampal neurons that induce an increased glutamate efflux blocked by a specific inhibitor (Miyamoto *et al*., 1998; Miyamoto *et al*., 2000).

The molecular basis for epsilon neurotoxicity is not known, but the toxin is a pore former, which structurally mimics *Aeromonas hydrophila* aerolysin (Cole *et al*., 2004) (see Chapter 35 of this volume). In Madin Darby Canine Kidney (MDCK) cells, which is a rare cell line sensitive to epsilon toxin, the toxin recognizes a specific unknown receptor, heptamerizes, and forms pores that lead to an efflux of $K^+$, influx of $Na^+$, $Cl^-$ and $Ca^{2+}$, cell swelling, and membrane blebbing/disruption (Petit *et al*., 1997; Petit *et al*., 2001; Miyata *et al*., 2002). In addition, epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells (Petit *et al*., 2003). Epsilon toxin also forms heptamers in synaptosomal membranes (Miyata *et al*., 2001; Miyata *et al*., 2002), which involves detergent-resistant membrane microdomains called lipid rafts, but it is not yet defined whether the toxin-induced glutamate release is due to pore formation or another specific mechanism.

## Toxins active on Rho-GTPases and possessing neurotoxicity

### *Toxins that inactivate Rho-GTPases*

Clostridial glucosylating toxins, also known as the large clostridial toxins, such as *Clostridium sordellii* lethal toxin (LT) and *Clostridium difficile* toxin B (ToxB), inactivate Rho-and/or Ras-GTPases by modifying a conserved threonine within the effector domain of target molecules (see Chapters 9 and 21, in this volume). ToxB catalyzes glucosylation of Rho proteins (Rho, Rac, and Cdc42), whereas the several variants of LT modify Rac, sometimes Cdc42, and various Ras proteins (i.e., Ras, Rap, Ral). *C. difficile* and *C. sordellii* toxins use UDP-glucose as cosubstrate, and *Clostridium novyi* α-toxin uses UDP-*N*-acetylglucosamine. GDP-bound GTPase is the preferred substrate, since the modified Thr35 and Thr37 residues are only exposed on the surface of this GTPase form. Although glucosylated GTPases can bind to their target membrane and undergo GDP-GTP exchange when stimulated by guanylate exchange factors, modification of Thr37/35 localized within switch I of GTPases prevents recognition with their effectors. In addition, intrinsic GAP (GTPase activating protein)-stimulated GTPase activity is reduced, inhibiting the GDP-GTP cycling of modified GTPases between the membrane and cytosol. Thereby, glycosylated GTPases act as dominant-negative mutants.

*C. botulinum* C3 enzyme is an ADP-ribosyltransferase, which specifically inactivates Rho (see Chapter 1 of this volume). C3 ADP-ribosylates Asn41, which is localized within the switch I loop near Thr37. C3-mediated ADP-ribosylation of Rho inhibits translocation to the membrane and therefore prevents interaction with its effectors. ADP-ribosylated Rho is trapped by guanine dissociation inhibitor (GDI) in an inactive, cytosolic form.

### *Neuronal alterations caused by toxin inactivation of Rho GTPases*

Several small GTPases of the Ras superfamily are present in nerve terminals and include RhoA, RhoB, Rac1, and Cdc42. Additionally, Rac1 associates with synaptic vesicles as well as the plasma membrane (Doussau *et al*., 2000; Humeau *et al*., 2002). The Ral molecule also binds to synaptic vesicles (Bielinski *et al*., 1993). Intraneuronal application of C3 exoenzyme, ToxB from *C. difficile*, or LT from *C. sordellii* (strain IP82 or VPI9048) blocks $Ca^{2+}$-dependent vesicular ACh release from *Aplysia* synapses (Doussau *et al*., 2000; Humeau *et al*., 2002). LT exhibits the highest activity upon neurotransmitter release, being 100- –1000- fold more potent than ToxB or the C3-exoenzyme from *C. botulinum* (Doussau *et al*., 2000). Similarly, LT blocks spontaneous glutamate release in cultured rat cerebellar slices (Kojima and Poulain, unpublished observation). These observations are consistent with reports that Rho-acting toxins block the secretory process in many cell types, including mast cells (Prepens *et al*., 1996; Kowluru *et al*., 1997; Gasman *et al*., 1999; Djouder *et al*., 2003). In muscles isolated from mice previously injected with LT, nerve-evoked muscle twitch is blocked. This effect results from both a presynaptic block of ACh release and depressed muscle contractility (Barbier *et al*., 2004). This is consistent with the high lethality of LT ($\sim 0.3 \times 10^6$ mouse $LD_{50}$/mg protein) injected intraperitoneally, which is probably due to respiratory failure. Because of the potent blocking action of LT on nerve endings, a question arises whether the intestinal disorders that characterize enterotoxemia caused by the clostridia producing Rho-acting toxins are linked to intestinal epithelial damage and/or stimulation of the enteric nervous system (Pothoulakis *et al*., 1998).

Identity of the downstream pathway whose silencing blocks exocytosis by LT or ToxB has been addressed in different cell systems. Apparently, the affected pathways can vary in different cell types. In several types of secretory cells, experimental findings indicate that the toxins induce remodeling of the actin cytoskeleton, thereby altering intracellular granule trafficking towards the plasma membrane, which possibly includes fusion (Prepens *et al*., 1996; Kowluru *et al*., 1997). Another interesting configuration has been deciphered in rat blood leukemia-mast cells. In these cells, exocytosis is triggered by a rise in intracellular $Ca^{2+}$ levels induced by stimulation of either the Fc-epsilon receptor or muscarinic-type AChR. The Rac GTPase is

the only one involved in $Ca^{2+}$ mobilization within these cells, and the blockade of exocytosis by ToxB or LT is due to inhibition of capacitative $Ca^{2+}$ entry exclusively through store-operated $Ca^{2+}$ channels (Djouder *et al*., 2000; Djouder *et al*., 2003). In nerve endings, the blockade of neurotransmitter exocytosis caused by ToxB and LT appears to have another origin. Studies performed with *Aplysia* cholinergic synapses reveal that following inactivation of Rac by LT or ToxB, the exocytosis process starts normally, but then stops, possibly during the priming events. This results in a dramatic decrease in the number of functional release sites (Humeau *et al*., 2002), which is very similar to the gross action of TeNT or BoNTs.

What is the role of Rac during priming? Rac, Rho, Cdc42, Ral, and PLD1 all participate in a multiprotein complex termed "exocyst," which is comprised of Sec6/Sec8 proteins and regulates polarized secretion as well as docking of vesicles to plasma membrane regions specialized in exocytosis (reviewed by Short and Barr, 2002). Therefore, one cannot exclude that Rho-acting toxins block exocytosis by disabling exocyst function in nerve endings. However, implication of an exocyst role during exocytosis of neurotransmitter is disputed. A likely possibility implicating PLD1 in the exocytosis process is that by catalyzing PA generation, formation of lipid bilayer intermediates becomes favored during membrane fusion. PLD1 activity is inhibited in LT-poisoned cells (Ben El Hadj *et al*., 1999), and Rac plus Cdc42 regulate PLD1 activity (Hammond *et al*., 1997). The intraneuronal application of a catalytically inactive mutant of PLD1 reproduces the potent inhibitory effect of LT (Humeau *et al*., 2001). Therefore, we envisage the following model for LT and ToxB action at the presynapse, which initially involves glucosylation of Rac by the toxins, thus preventing activation of PLD1 upon synaptic vesicle docking. Production of fusogenic PA is not stimulated, and despite the $Ca^{2+}$ influx triggering SNARE complex zippering, fusion of synaptic vesicle with plasmalemma cannot occur because the lipid composition at release sites remains inadequate. The synaptic vesicles equipped with modified Rac plug exocytosis sites and prevent fusion of synaptic vesicles equipped with intact Rac. This contributes to reduced numbers of active release sites by LT or ToxB.

In addition to the presynaptic actions mentioned above, clostridial cytotoxins affecting Rho GTPases can also block neurotransmission by altering postsynaptic cell capabilities to detect transmitter. For example, neurotransmitter-receptor density is diminished in neurons treated with toxins that affect Rac or Cdc42 (Meyer *et al*., 2000). The observation that LT prevents long-term potentiation (Murray and O'Connor, 2004) is consistent with inhibiting insertion of new receptors into the post-synaptic membrane. Note that it is not yet clear whether changes in receptor density refer to the role of Rho proteins during the exocytotic process, which includes receptor insertion into the plasma membrane, and/or organization of the post-synaptic actin cytoskeleton involved in receptor clustering (Triller and Choquet, 2003).

Much evidence suggests that apoptosis plays a crucial role in cell homeostasis, which depends upon the expression of various genes implicated in controlling life and death. Incubation of rat primary cerebellar granule neurons in culture with either ToxB or LT reportedly induces cell death with biochemical and morphological hallmarks of neuronal apoptosis (Linseman *et al*., 2001). In these cells, selective inhibition of Rac/Cdc42 function promotes: (i) phosphorylation and expression of the transcription factor c-Jun; (ii) activation of caspase-3; and (iii) nuclear condensation, as well as fragmentation. Interestingly, apoptosis occurs independently of F-actin-cytoskeletal disruption, since agents that directly disassemble F-actin (*C. botulinum* C2 toxin, cytochalasin D, and latrunculin A) do not induce cell death within 24 h. These results indicate that Rac/Cdc42 GTPases are critical for survival of cerebellar granule neurons in primary culture. Additionally, these data are the first to establish a prosurvival function for Rho GTPases in a primary neuronal cell model.

## ENTEROTOXINS AND THE ENTERIC NERVOUS SYSTEM

Increasing evidence suggests that some enterotoxins mediate diarrhea by not only acting directly upon enterocytes, but also by interfering with/stimulating the enteric nervous system (Pothoulakis *et al*., 1998). The enteric nervous system is comprised of the myenteric plexus involved primarily in motor control of the gut and the submucosal plexus, which plays a central role in sensing the chemical status of the intestine and regulating secretion. Neuronal cell bodies of the enteric nervous system (intrinsic afferent neurons, interneurons, motor- and secretory-neurons) are clustered in ganglia and connected to parasympathetic and sympathetic systems. More than 20 neurotransmitters have been identified in the enteric nervous system. Among them, substance P, ACh, GABA, glutamate, and 5HT modulate motility, absorption, and secretion of the intestine (Farthing, 2000; Farthing *et al*., 2004).

Cholera toxin (CT) is well known to mono-ADP-ribosylate heterotrimeric G-proteins leading to adenylcyclase stimulation, increased intracellular levels of cAMP, plus

$Cl^-$ and water secretion from the cell into the intestinal lumen (see Chapter 15 of this volume). In addition, CT stimulates 5HT release from entero chromaffin cells localized to the villus of the small intestine. Although the cascade-response involving the enteric nervous system is still unclear, it is suggested that 5HT activates afferent nerves in the lamina propria containing ACh or substance P as neurotransmitter, which subsequently stimulate secretory neurons releasing vasoactive intestinal polypeptide (VIP) and possibly NO. VIP recognizes specific receptors on the basolateral membrane of crypt cells and induces an adenyl cyclase-cAMP-dependent secretion of NaCl and water. Antagonists of 5HT, substance P, and VIP markedly reduce CT-induced secretion (Farthing, 2000).

The *Escherichia coli* heat labile enterotoxin (HLT) is structurally related to CT, sharing a classic 1A (enzyme component):5B (cell binding component) structure of approximately 85 kDa; however, HLT does not stimulate the release of (5HT?) from enterochromaffin cells. HLT-induced secretion is not inhibited by substance P, but lignocaine and the ganglionic blocker hexamethonium have a preventive effect, suggesting that the enteric nervous system is involved in the enteric activity of HLT (Farthing, 2000).

The much smaller *E. coli* heat stable enterotoxins (ST; 2–5 kDa) activate guanylate cyclase, increase cyclic guanosine monophosphate (cGMP) levels, and subsequently open $Cl^-$ channels (see Chapter 48 of this volume). Since ST-induced secretion is blocked by tetrodotoxin, lignocaine, and hexamethonium, the entero nervous system also likely supports ST activity (Farthing, 2000).

*C. difficile* toxin A (ToxA; ~300 kDa) is a potent enterotoxin responsible for pseudomembranous colitis (see Chapter 21 of this volume). In addition to the apparent actin-cytoskeleton alterations in enterocytes, ToxA probably uses a neural mechanism to increase intestinal secretion. Lignocaine, hexamethonium, and substance P inhibitors can block Tox A-induced ileal fluid accumulation, mucosal permeability, and inflammation (Castagliuolo *et al*., 1994). It is proposed that ToxA stimulates primary sensory neurons in the intestinal mucosa, causing subsequent release of substance P and CGRP, which then facilitates intestinal secretion and inflammation. These neuropeptides bind to macrophages in the lamina propria and activate the release of proinflammatory cytokines such as tumor necrosis factor (TNF) (Pothoulakis and Lamont, 2001). Thereby, ToxA stimulates intestinal macrophage expression of neurokinin-1 (NK-1), which is a high affinity receptor for substance P, and additionally NK-1 deficient mice are protected from ToxA-induced intestinal secretion and inflammatory diarrhea (Castagliuolo *et al*., 1998). Neurotensin, a gastrointestinal neuropeptide, is also involved since an inhibitor of neurotensin prevents the secretory and inflammatory responses following ToxA exposure (Castagliuolo *et al*., 1999). Furthermore, ToxA also suppresses noradrenaline release at sympathetic synapses of the enteric nervous system that play an inhibitory effect on secretomotor neurons, thus amplifying mucosal secretion (Xia *et al*., 2000).

Finally, in addition to the various enterotoxins from bacilli listed above, *Staphylococcus aureus* also produces a myriad of 25–30 kDa, single-chain proteins called the staphylococcal enterotoxins (SEs) (Krakauer and Stiles, 2003). These potent toxins cause a prevalent form of food poisoning found throughout the world, and these molecules also possess "superantigenic" properties (see Chapter 49 of this volume). By classic definition, this latter property involves binding to both major histocompatibility complex class II (MHC II) on antigen presenting cells and Vβ specific T-cell receptors, which ultimately causes massive T-cell proliferation with a concomitant production/release of various proinflammatory cytokines. To date, it is uncertain if superantigenicity plays a direct role in SE-induced food poisoning. However, levels of inflammatory mediators like prostaglandins and leukotrienes are increasingly evident in the circulatory system of nonhuman primates shortly after an oral dose of SEB (Jett *et al*., 1990). Mast cells may also play a role in SE-induced food poisoning, which perhaps involves not only inflammatory mediators but also stimulation by neuropeptides like substance P released from sensory neurons (Alber *et al*., 1989). Another study reveals that SEB-induced effects in mice (intraperitoneal injection) are abrogated by capsaicin, the active ingredient of hot chili peppers, which depletes peptidergic sensory nerve fibers and TNF production (Tiegs *et al*., 1999). An intraperitoneal injection of SEB into rats induces expression of Fos (a cell activator) throughout the brain via vagus nerve stimulation, thus suggesting that the peripheral presence of an SE has profound effects upon the brain (Wang *et al*., 2004). Similar results (capsaicin inhibition and vagal nerve stimulation) have also been reported for *E. coli* ST in rats (Rolfe and Levin, 1999). Exactly how an SE molecule communicates directly (or indirectly) with the vagus nerve is unknown, but MHC II-bearing cells found between fibrous vagus bundles in the abdomen may provide a clue that involves interleukin 1β (Goehler *et al*., 1999). All of this evidence indirectly suggests a role for the nervous system during SE enterotoxicity, but in our opinion, more definitive studies await.

## CONCLUSION

Is neurotoxicity a unique, inherent function of a bacterial toxin? Most bacterial proteins with demonstrable "toxic" activity, such as those presented in this chapter, interact with various cell types. For example, more than one-third of all bacterial toxins are pore-formers that recognize ubiquitous membrane components as receptors, such as cholesterol, gangliosides, and proteins. These toxins can indiscriminately damage membranes from different cells, including those of neuronal origin. Among them, *C. perfringens* epsilon toxin has the fundamental structure of a pore-forming toxin, is cytotoxic for kidney epithelial cells, and also possesses specific neurotoxic activity. However, a specific neurotoxic mechanism of action and trafficking pattern targeting neuronal cells by epsilon toxin during the natural course of disease still remains undetermined. Other toxins have developed various internalization processes, therefore specifically modifying an intracellular target. Thus, several bacterial proteins like the large clostridial toxins (i.e., *C. sordellii* LT and *C. difficile* ToxA/ToxB) have the ability to bind/enter various cell types that include those of neuronal origin. Large clostridial toxins inactivate intracellular targets like Rho and/or Ras GTPases, which are involved in multiple signaling pathways. Among them, Rac controls the neuroexocytosis process. Thereby, large clostridial toxins efficiently block neurotransmitter release, although they do not specifically target neuronal cells. Involvement of their neurotoxic activity during disease, which is often overcome by toxic effects upon other cell types in natural pathology, is also a function of a toxin's opportunity to enter neuronal cells near the infection site. Finally, two unique classes of neurotoxins, the BoNTs and TeNT, have evolved as specific inhibitors of the neuroexocytosis machinery. These bacterial proteins recognize specific receptors on neuronal cells and only interfere intracellularly with highly specialized molecules, like the SNARE proteins, which play a pivotal role in evoked release of neurotransmitter. It is intriguing that toxins produced by environmental bacteria, which are not normally adapted for a commensal life with higher organisms and only interact accidentally with them, possess such specific and highly sophisticated tools. What is the underlying selective pressure for such evolution from a bacterial protease to a neurotoxin? Perhaps neurotoxins simply result from an evolutionary process independent of the host. What is the inherit benefit derived by an environmental bacterium to produce a neurotoxin, which apparently does not recognize other bacterial or environmental substrates? Clearly, we approach this from a scientist's (human) perspective, and with such an inherent bias it may be difficult and virtually impossible to truly understand this natural curiosity.

During their evolution, clostridial pathogens have developed a potent arsenal of noxious proteins that affect the central and peripheral nervous system of various vertebrates. These commonly named neurotoxins specifically target some key functions, or cellular processes, of eukaryotic cells, which subsequently cause a wide array of life-threatening diseases in humans and animals. These neurotoxins bind to specific receptors in the plasma membrane of susceptible host cells and translocate their enzymatically active domains/subunits into the cytosol, where finally they elicit the deleterious effects commonly associated with the holotoxins. Modes of action for the neurotoxic proteins reviewed in this chapter have been deciphered during the last decade, and now a clearer picture of their intracellular substrates is emerging. The specificity of these toxic proteins has enabled them to become useful tools to elucidate and characterize crucial processes for eukaryotic cells, which include neurotransmitter release, physiological signaling pathways, and constitutive cellular mechanisms. However, additional studies are needed to completely identify their specific membrane-receptors, as well as comprehend the mechanisms and structures involved in toxin routing throughout the nervous system. Thus, multidisciplinary approaches integrating molecular microbiology, membrane biology, biochemistry, physiology, proteomics, and pharmacology will further advance our understanding of the transport mechanisms required for directing molecules to specific locations within the nervous system of eukaryotes. Clearly, specificity of action for the BoNTs has made them very useful therapeutic agents for many human neurological syndromes caused by hyperactivity of cholinergic nerve terminals (see Chapter 58, in this volume). It is expected that future pharmacological developments will employ the inherent capabilities of various clostridial neurotoxins to deliver biologically active substances into nerve cells.

## REFERENCES

Abdipranoto, A., Liu, G.J., Werry, E.L. and Bennett, M.R. (2003). Mechanisms of secretion of ATP from cortical astrocytes triggered by uridine triphosphate. *Neuroreport* **14,** 2177–2181.

Alber, G., Scheuber, P.H., Reck, B., Sailer-Kramer, B., Hartmann, A. and Hammer, D.K. (1989). Role of substance P in immediate-type skin reactions induced by staphylococcal enterotoxin B in unsensitized monkeys. *J. Allergy. Clin. Immunol*. **84,** 880–885.

Alderson, K., Yee, W.C. and Pestronk, A. (1989). Reorganization of intrinsic components in the distal motor axon during outgrowth. *J. Neurocytol*. **18,** 541–552.

Aleu, J., Blasi J, Solsona C and J., M. (2002). Calcium-dependent acetylcholine release from Xenopus oocytes: simultaneous ionic currents and acetylcholine release recordings. *Eur. J. Neurochem*. **8,** 1442–1448.

Angaut-Petit, D., Molgo, J., Comella, J.X., Faille, L. and Tabti, N. (1990). Terminal sprouting in mouse neuromuscular junctions poisoned with botulinum type A toxin: morphological and electrophysiological features. *Neuroscience* **37,** 799–808.

Antharavally, B.S. and DasGupta, B.R. (1997). Covalent structure of botulinum neurotoxin type E: location of sulfhydryl groups and disulfide bridges and identification of C-termini of light and heavy chains. *J. Protein Chem*. **16,** 787–799.

Araque, A., Li, N., Doyle, R.T. and Haydon, P.G. (2000). SNARE protein-dependent glutamate release from astrocytes. *J. Neurosci*. **20,** 666–673.

Arimitsu, H., Inoue, K., Sakaguchi, Y., Lee, J., Fujinaga, Y., Watanabe, T., Ohyama, T., Hirst, R. and Oguma, K. (2003). Purification of fully activated *Clostridium botulinum* serotype B toxin for treatment of patients with dystonia. *Infect. Immun*. **71,** 1599–1603.

Arndt, J.W., Gu, J., Jaroszewski, L., Schwarzenbacher, R., Hanson, M.A., Lebeda, F.J. and Stevens, R.C. (2005). The structure of the neurotoxin-associated protein HA33/A from *Clostridium botulinum* suggests a reoccurring beta-trefoil fold in the progenitor toxin complex. *J. Mol. Biol*. **346,** 1083–1093.

Ashton, A.C., Li, Y., Doussau, F., Weller, U., Dougan, G., Poulain, B. and Dolly, O. (1995). Tetanus toxin inhibits neuroexocytosis even when its $Zn^{2+}$-dependent protease activity is removed. *J. Biol. Chem*. **270,** 31386–31390.

Atlas, D. (2001). Functional and physical coupling of voltage-sensitive calcium channels with exocytotic proteins: ramifications for the secretion mechanism. *J. Neurochem*. **77,** 972–985.

Augustine, G.J. (2001). How does calcium trigger neurotransmitter release? *Curr. Opin. Neurobiol*. **11,** 320–326.

Aureli, P., Fenicia, L., Pasolini, B., Gianfranceschi, M., McCroskey, L.M. and Hatheway, C.L. (1986). Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. *J. Infect. Dis*. **154,** 207–211.

Bader, M.F., Doussau, F., Chasserot-Golaz, S., N., V. and Gasman, S. (2004). Coupling actin and membrane dynamics during calcium-regulated exocytosis: a role for Rho and ARF GTPases. *Biochim. Biophys. Acta*. **1742,** 37–49.

Bajohrs, M., Rickman, C., Binz, T. and Davletov, B. (2004). A molecular basis underlying differences in the toxicity of botulinum serotypes A and E. *EMBO Rep*. **5,** 1090–1095.

Baldwin, M.R., Bradshaw, M., Johnson, E.A. and Barbieri, J.T. (2004). The C-terminus of botulinum neurotoxin type A light chain contributes to solubility, catalysis, and stability. *Protein Express. Purif*. **37,** 187–195.

Bambrick, L. and Gordon, T. (1987). Acetylcholine receptors and sodium channels in denervated and botulinum-toxin-treated adult rat muscle. *J. Physiol*. **382,** 69–86.

Bambrick, L.L. and Gordon, T. (1992). Neural regulation of acetylcholine receptors in rat neonatal muscle. *J. Physiol*. **449,** 479–492.

Barbier, J., Popoff, M.R. and Molgo, J. (2004). Degeneration and regeneration of murine skeletal neuromuscular junctions after intramuscular injection with a sublethal dose of *Clostridium sordellii* lethal toxin. *Infect. Immun*. **72,** 3120–3128.

Ben El Hadj, N., Popoff, M.R., Marvaud, J.C., Payrastre, B., Boquet, P. and Geny, B. (1999). G-protein-stimulated phospholipase D activity is inhibited by lethal toxin from *Clostridium sordellii* in HL-60 cells. *J. Biol. Chem*. **274,** 14021–14031.

Benke, T.A. and Swann, J. (2004). The tetanus toxin model of chronic epilepsy. *Adv. Exp. Med. Biol*. **548,** 226–238.

Bergsman, J.B. and Tsien, R.W. (2000). Syntaxin modulation of calcium channels in cortical synaptosomes as revealed by botulinum toxin C1. *J. Neurosci*. **20,** 4368–4378.

Bielinski, D.F., Pyun, H.Y., Linko-Stentz, K., Macara, I.G. and Fine, R.E. (1993). Protein Ral and Rab3a are major GTP-binding proteins of axonal rapid transport and synaptic vesicles and do not redistribute following depolarization stimulated synaptosomal exocytosis. *Biochim. Biophys. Acta*. **1151,** 246–256.

Bigalke, H. and Shoer, L.F. (2000). Clostridial neurotoxins. In: *Bacterial Protein Toxins*, (eds. K. Aktories and I. Just), pp. 407–443 Springer, Berlin.

Binz, T., Bade, S., Rummel, A., Kollewe, A. and Alves, J. (2002). Arg(326) and Tyr(365) of the botulinum neurotoxin type A light chain are involved in transition state stabilization. *Biochemistry* **41,** 1717–1723.

Bleck, T.P. (1989). Clinical aspects of tetanus. In: *Botulinum Neurotoxin and Tetanus Toxin*, (ed. L.L. Simpson), pp. 379–398. Academic Press, San Diego.

Brin, M.F., Hallett, M. and Jankovic, J. (2002). *Scientific and Therapeutic Aspects of Botulinum Toxin*. Lippincott, Williams, and Wilkins: Philadelphia USA.

Brisinda, G., Bentivoglio, A.R., Maria, G. and Albanese, A. (2004). Treatment with botulinum neurotoxin of gastrointestinal smooth muscles and sphincter spasms. *Mov. Disord*. **19,** S146–S156.

Brose, N., Rosenmund, C. and Rettig, J. (2000). Regulation of transmitter release by Unc-13 and its homologues. *Curr. Opin. Neurobiol*. **10,** 303–311.

Brown, M.C., Holland, R.L. and Hopkins, W.G. (1981). Motor nerve sprouting. *Annu. Rev. Neurosci*. **4,** 17–42.

Bruns, D., Engers, S., Yang, C., Ossig, R., Jeromin, A. and Jahn, R. (1997). Inhibition of transmitter release correlates with the proteolytic activity of tetanus toxin and botulinum toxin A in individual cultured synapses of *Hirudo medicinalis*. *J. Neurosci*. **17,** 1898–1910.

Brüggemann, H., Bäumer, S., Fricke, W.F., Wiezr, A., Liesagang, H., Decker, I., Herzberg, C., Martinez-Arias, R., Henne, A. and Gottschalk, G. (2003). The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proc. Natl. Acad. Sci. USA* **100,** 1316–1321.

Buffelli, M., Pasino, E. and Cangiano, A. (2001). *In vivo* acetylcholine receptor expression induced by calcitonin gene-related peptide in rat soleus muscle. *Neuroscience* **104,** 561–567.

Bullough, P.A., Hughson, F.M., Skehel, J.J. and Wiley, D.C. (1994). Structure of influenza hemagglutinin at the pH of membrane fusion. *Nature (London)* **371,** 37–43.

Burden, S.J. (1993). Synapse-specific gene expression. *Trends Genet*. **9,** 12–16.

Buxton, D. (1978). The use of an imunoperoxidase technique to investigate by light and electron microscopy the sites of binding of *Clostridium welchii* type D ε-toxin in mice. *J. Med. Microbiol*. **11,** 289–292.

Cabot, J.B., Mennone, A., Bogan, N., Carroll, J., Evinger, C. and Erichsen, J.T. (1991). Retrograde, trans-synaptic and transneuronal transport of fragment C of tetanus toxin by sympathetic preganglionic neurons. *Neurosci*. **40,** 805–823.

Caccin, P., Rossetto, O., Rigoni, M., Johnson, E.A., Schiavo, G. and Montecucco, C. (2003). VAMP/synaptobrevin cleavage by tetanus and botulinum neurotoxins is strongly enhanced by acidic liposomes. *FEBS Lett*. **542,** 132–136.

Cai, S., Sarkar, H.K. and Singh, B.R. (1999). Enhancement of the endopeptidase activity of botulinum neurotoxin by its associated proteins and dithiothreitol. *Biochemistry* **38,** 6903–6910.

Cai, S. and Singh, B.R. (2001). Role of the disulfide cleavage induced molten globule state of type A botulinum neurotoxin in its endopeptidase activity. *Biochemistry* **40,** 15327–15333.

Capogna, M., McKinney, R.A., O'Connor, V., Gahwiler, B.H. and Thompson, S.M. (1997). $Ca^{2+}$ or $Sr^{2+}$ partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin. *J. Neurosci*. **17,** 7190–7202.

Caroni, P., Schneider, C., Kiefer, M. and Zapf, J. (1994). Role of muscle insulin-like growth factors in nerve sprouting: suppression of terminal sprouting in paralyzed muscle by IGF-binding protein 4. *J. Cell Biol*. **125,** 893–902.

Castagliuolo, I., Lamont, J.T., Letourneau, R., Kelly, C., O'Keane, J.C., Jaffer, A., Theoharides, T.C. and Pothoulakis, C. (1994). Neuronal involvement in the intestinal effects of *Clostridium difficile* toxin A and *Vibrio cholerae* enterotoxin in rat ileum. *Gastroenterol*. **107,** 657–665.

Castagliuolo, I., Riegler, M., Pasha, A., Nikulasson, S., Lu, B., Gerard, C., Gerard, N.P. and Pothoulakis, C. (1998). Neurokinin-1 (NK-1) receptor is required in *Clostridium difficile*-induced enteritis. *J. Clin. Invest*. **101,** 1547–1550.

Castagliuolo, I., Wang, C.C., Valenick, L., Pasha, A., Nikulasson, S., Carraway, R.E. and Pothoulakis, C. (1999). Neurotensin is a proinflammatory peptide in colonic inflammation. *J. Clin. Invest*. **103,** 843–849.

Chaib-Oukadour, I., Gil, C. and Aguilera, J. (2004). The C-terminal domain of the heavy chain of tetanus toxin rescues cerebellar granule neurones from apoptotic death: involvement of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. *J. Neurochem*. **90,** 1227–1236.

Chapman, E.R. (2002). Synaptotagmin: a $Ca^{(2+)}$ sensor that triggers exocytosis? *Nat. Rev. Mol. Cell Biol*. **3,** 498–508.

Chen, F., Kuziemko, G.M. and Stevens, R.C. (1998). Biophysical characterization of the stability of the 150-kilodalton botulinum toxin, the nontoxic component, and the 900-kilodalton botulinum toxin complex species. *Infect. Immun*. **66,** 2420–2425.

Chen, Y.A., Scales, S.J., Jagath, J.R. and Scheller, R.H. (2001). A discontinuous SNAP-25 C-terminal coil supports exocytosis. *J. Biol. Chem*. **276,** 28503–28508.

Chen, Y.A., Scales, S.J., Patel, S.M., Doung, Y.C. and Scheller, R.H. (1999). SNARE complex formation is triggered by $Ca^{2+}$ and drives membrane fusion. *Cell* **97,** 165–174.

Cheng, L., Khan, M. and Mudge, A.W. (1995). Calcitonin gene-related peptide promotes Schwann cell proliferation. *J. Cell Biol*. **129,** 789–796.

Chernomordik, L.V. and Kozlov, M.M. (2003). Protein-lipid interplay in fusion and fission of biological membranes. *Annu. Rev. Biochem*. **72,** 175–207.

Choi, W.S., Kim, Y.M., Combs, C., Frohman, M.A. and Beaven, M.A. (2002). Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. *J. Immunol*. **168,** 5682–5689.

Coen, L., Osta, R., Maury, M. and Brulet, P. (1997). Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous sytem. *Proc. Natl. Acad. Sci. (USA)* **94,** 9400–9405.

Coffield, J.A., Bakry, N., Zhang, R.D., Carlson, J., Gomella, L.G. and Simpson, L.L. (1997). *In vitro* characterization of botulinum toxin types A, C, and D action on human tissues: combined electrophysiology, pharmacologic, and molecular biologic approaches. *J. Pharmacol. Exp. Ther*. **280,** 1489–1498.

Coffield, J.A., Bakry, N.M., Maksymowych, A.B. and Simpson, L.L. (1999). Characterization of a vertebrate neuromuscular junction that demonstrates selective resistance to botulinum toxin. *J. Pharmacol. Exp. Ther*. **289,** 1509–1516.

Cohen, R. and Atlas, D. (2004). R-type voltage-gated $Ca^{(2+)}$ channel interacts with synaptic proteins and recruits synaptotagmin to the plasma membrane of Xenopus oocytes. *Neuroscience* **128,** 831–841.

Cole, A.R., Gibert, M., Popoff, M.R., Moss, D.S., Titball, R.W. and Basak, A. (2004). *Clostridium perfringens* β-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat. Struct. Mol. Biol*. **11,** 797–798.

Comella, J.X., Molgo, J. and Faille, L. (1993). Sprouting of mammalian motor nerve terminals induced by in vivo injection of botulinum type-D toxin and the functional recovery of paralysed neuromuscular junctions. *Neurosci. Lett*. **153,** 61–64.

Cordivari, C., Misra, V.P., Catania, S. and Lees, A.J. (2004). New therapeutic indications for botulinum toxins. *Mov. Disord*. **S8,** S157–S161.

Cornille, F., Deloye, F., Fournie-Zaluski, M.C., Roques, B.P. and Poulain, B. (1995). Inhibition of neurotransmitter release by synthetic proline-rich peptides shows that the N-terminal domain of vesicle-associated membrane protein/synaptobrevin is critical for neuro-exocytosis. *J. Biol. Chem*. **270,** 16826–16832.

Cornille, F., Martin, L., Lenoir, C., Cussac, D., Doques, B.P. and Fournie-Zaluski, M.C. (1997). Cooperative exosite-dependent cleavage of synaptobrevin by tetanus toxin light chain. *J. Biol. Chem*. **272,** 3459–3464.

Cremona, O. and De Camilli, P. (2001). Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci*. **114,** 1041–1052.

Curra, A., Trompetto, C., Abbruzzese, G. and Berardelli, A. (2004). Central effects of botulinum toxin type A: evidence and supposition. *Mov. Disord*. **19,** S60–S64.

DasGupta, B.R. and Tepp, W. (1993). Protease activity of botulinum neurotoxin type E and its light chain: cleavage of actin. *Biochem. Biophys. Res. Commun*. **190,** 470–474.

de Haro, L., Ferracci, G., Opi, S., Iborra, C., Quetglas, S., Miquelis, R., Leveque, C. and Seagar, M. (2004). $Ca^{2+}$/calmodulin transfers the membrane-proximal lipid-binding domain of the v-SNARE synaptobrevin from cis to trans bilayers. *Proc. Natl. Acad. Sci. (USA)* **101,** 1578–1583.

de Paiva, A., Meunier, F., Molgo, J., Aoki, K.R. and Dolly, J.O. (1999). Functional repair of motor endpates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. *Proc. Natl. Acad. Sci. (USA)* **96,** 3200–3205.

Degtiar, V.E., Scheller, R.H. and Tsien, R.W. (2000). Syntaxin modulation of slow inactivation of N-type calcium channels. *J. Neurochem*. **20,** 4355–4367.

Dineen, S.S., Bradshaw, M. and Johnson, E.A. (2003). Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. *Cur. Microbiol*. **46,** 342–352.

Djouder, N., Aneiros, E., Cavalie, A. and Aktories, K. (2003). Effects of large clostridial cytotoxins on activation of RBL 2H3-hm1 mast cells indicate common and different roles of Rac in FcepsilonRI and M1-receptor signaling. *J. Pharmacol. Exp. Ther*. **304,** 1243–1250.

Djouder, N., Prepens, U., Aktories, K. and Cavalie, A. (2000). Inhibition of calcium release-activated calcium current by Rac/Cdc42-inactivating clostridial cytotoxins in RBL cells. *J. Biol. Chem*. **275,** 18732–18738.

Doussau, F. and Augustine, G.J. (2000). The actin cytoskeleton and neurotransmitter release: an overview. *Biochimie* **82,** 353–363.

Doussau, F., Gasman, S., Humeau, Y., Vitiello, F., Popoff, M.R., Boquet, P., Bader, M.F. and Poulain, B. (2000). A Rho-related GTPase is involved in $Ca^{++}$-dependent neurotransmitter exocytosis. *J. Biol. Chem*. **275,** 7764–7770.

Dressler, D. and Benecke, R. (2003). Autonomic side effects of botulinum toxin type B treatment of cervical dystonia and hyperhydrosis. *Eur. Neurol*. **49,** 34–38.

Durham, P.L., Cady, R. and Cady, R. (2004). Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by bot-

ulinum toxin type A: implications for migraine therapy. *Headache* **44,** 35–42.

Eisel, U., Reynolds, K., Riddick, M., Zimmer, A., Niemann, H. and Zimmer, A. (1993). Tetanus toxin light chain expression in Sertoli cells of transgenic mice causes alterations of the actin cytoskeleton and disrupts spermatogenesis. *EMBO J.* **12,** 3365–3372.

Eitzen, G. (2003). Actin remodeling to facilitate membrane fusion. *Biochim. Biophys. Acta*. **1641,** 175–181.

Eleopra, R., Tugnoli, V., Rossetto, O., De Grandis, D. and Montecucco, C. (1998). Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. *Neurosci. Lett*. **256,** 135–138.

Emsley, P., Fotinou, C., Black, I., Fairweather, N.F., Charles, I.G., Watts, C., Hewitt, E. and Isaacks, N.W. (2000). The structures of the Hc fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding. *J. Biol. Chem*. **275,** 8889–8894.

Farthing, M.J.G. (2000). Enterotoxins and the enteric nervous system—a fatal attraction. *Int. J. Med. Microbiol*. **290,** 491–496.

Farthing, M.J.G., Casburn-Jones, A. and Banks, M.R. (2004). Enterotoxins, enteric nerves, and intestinal secretion. *Cur. Gastroenterology Rep*. **6,** 177–180.

Fasshauer, D. and Margittai, M. (2004). A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. *J. Biol. Chem*. **279,** 7613–7621.

Fenicia, L., Franciosa, G., Pourshaban, M. and Aureli, P. (1999). Intestinal toxemia botulism in two young people caused by *Clostridium butyricum* Type E. *Clin. Infect. Dis*. **29,** 381–387.

Fernandez-Salas, E., Steward, L.E., Ho, H., Garay, P.E., Sun, S.W., Gilmore, M.A., Ordas, J.V., Wang, J., Francis, J. and Aoki, K.R. (2004). Plasma membrane localization signals in the light chain of botulinum neurotoxin. *Proc. Natl. Acad. Sci. USA* **101,** 3208–3213.

Ferrer-Montiel, A.V., Gutierrez, L.M., Apland, J.P., Canaves, J.M., Gil, A., Viniegra, S., Biser, J.A., Adler, M. and Montal, M. (1998). The 26-mer peptide released from SNAP-25 cleavage by botulinum neurotoxin E inhibits vesicle docking. *FEBS Lett*. **435,** 84–88.

Fili, O., Michaelevsk, I., Bledi, Y., Chikvashvili, D., Singer-Lahat, D., Boshwitz, H., Linial, M. and Lotan, I. (2001). Direct interaction of a brain voltage-gated K+ channel with syntaxin 1A: functional impact on channel gating. *J. Neurochem*. **21,** 1964–1974.

Filippi, G.M., Errico, P., Santarelli, R., Bagolini, B. and Manni, E. (1993). Botulinum A toxin effects on rat jaw muscle spindles. *Acta Otolaryngol*. **113,** 400–404.

Finnie, J.W., Blumbergs, P.C. and Manavis, J. (1999). Neuronal damage produced in rat brains by *Clostridium perfringens* type D epsilon-toxin. *J. Comp. Path*. **120,** 415–420.

Fontaine, B., Klarsfeld, A. and Changeux, J.P. (1987). Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol*. **105,** 1337–1342.

Foran, P.G., Mohammed, N., Lisk, G.O., Nagwaney, S., Lawrence, G.W., Johnson, E., Smith, L., Aoki, K.R. and Dolly, O.J. (2003). Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. *J. Biol. Chem*. **278,** 1363–1371.

Fotinou, C., Emsley, P., Black, I., Ando, H., Ishida, H., Kiso, M., Sinha, K.A., Fairweather, N.F. and Isaacs, N.W. (2001). The crystal structure of tetanus toxin Hc fragment complexed with a synthetic GT1b analog suggests cross-linking between ganglioside receptors and the toxin. *J. Biol. Chem*. **276,** 3274–3281.

Fu, F.N., Sharma, S.K. and Singh, B.R. (1997). A protease-resistant novel hemagglutinin purified from type A *Clostridium botulinum. J. Prot. Chem*. **17,** 53–60.

Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvaud, J.C., Popoff, M.R., Kozaki, S. and Oguma, K. (2000). Identification and characterization of functional subunits of *Clostridium botulinum* A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. *FEBS Lett*. **467,** 179–183.

Fujinaga, Y., Inoue, K., Watanabe, S., Yokota, K., Hirai, Y., Nagamachi, E. and Oguma, K. (1997). The hemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig intestine, leading to the efficient absorption of the toxin. *Microbiol*ogy **143,** 3841–3847.

Fujinaga, Y., Inoue, K., Watarai, S., Sakaguchi, G., Arimitsu, H., Lee, J.C., Jin, Y., Matsumura, T., Kabumoto, Y., Watanabe, T., Ohyama, T., Nishikawa, A. and Oguma, K. (2004). Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes. *Microbiol*. **150,** 1529–1538.

Fujita, R., Fujinaga, Y., Inoue, K., Nakajima, H., Kumon, H. and Oguma, K. (1995). Molecular characterization of two forms of non-toxic, non-hemagglutinantinin components of *Clostridium botulinum* type A progenitor toxins. *FEBS Lett*. **376,** 41–44.

Galli, T. and Haucke, V. (2004). Cycling of synaptic vesicles: how far? How fast! *Sci.* STKE. **264,** RE 19.

Gallwitz, D. and Jahn, R. (2003). The riddle of the Sec1/Munc-18 proteins—new twists added to their interactions with SNAREs. *Trends Biochem. Sci*. **28,** 113–116.

Garner, C.C., Kindler, S. and Gundelfinger, E.D. (2000). Molecular determinants of presynaptic active zones. *Curr. Opin. Neurobiol*. **10,** 321–327.

Gasman, S., Chasserot-Golaz, S., Malacombe, M., Way, M. and Bader, M.F. (2004). Regulated exocytosis in neuroendocrine cells: a role for subplasmalemmal Cdc42/N-WASP-induced actin filaments. *Mol. Biol. Cell* **15,** 520–531.

Gasman, S., Chasserot-Golaz, S., Popoff, M.R., Aunis, D. and Bader, M.F. (1999). Involvement of Rho GTPases in calcium-regulated exocytosis from adrenal chromaffin cells. *J. Cell Sci*. **112,** 4763–4771.

Georgiou, J., Robitaille, R., Trimble, W.S. and Charlton, M.P. (1994). Synaptic regulation of glial protein expression *in vivo. Neuron* **12,** 443–455.

Gerona, R.R., Larsen, E.C., Kowalchyk, J.A. and Martin, T.F. (2000). The C terminus of SNAP25 is essential for $Ca^{2+}$–dependent binding of synaptotagmin to SNARE complexes. *J. Biol. Chem*. **275,** 6328–6336.

Gil, C., Chaib-Oukadour, I. and Aguilera, J. (2003). C-terminal fragment of tetanus toxin heavy chain activates Akt and MEK/ERK signaling pathways in a Trk receptor-dependent manner in cultured cortical neurons. *Biochem. J.* **15,** 613–620.

Goehler, L.E., Gaykema, R.P., Nguyen, K.T., Lee, J.E., Tilders, F.J., Maier, S.F. and Watkins, L.R. (1999). Interleukin-1beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? *J. Neurosci*. **19,** 2799–2806.

Gonzalez-Forero, D., de la Cruz, R.R., Delgado-Garcia, J.M., Alvarez, F.J. and Pastor, A.M. (2003). Functional alterations of cat abducens neurons after peripheral tetanus neurotoxin injection. *J. Neurophysiol*. **89,** 1878–1890.

Gonzalez-Forero, D., Pastor, A.M., Delgado-Garcia, J.M., de la Cruz, R.R. and Alvarez, F.J. (2004). Synaptic structural modification following changes in activity induced by tetanus neurotoxin in cat abducens neurons. *J. Comp. Neurol*. **471,** 201–218.

Graham, M.E., Fisher, R.J. and Burgoyne, R.D. (2000). Measurement of exocytosis by amperometry in adrenal chromaffin cells: effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. *Biochimie* **82,** 469–479.

Habermann, E. and Dreyer, F. (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. *Cur. Top. Microbiol. Immunol*. **129,** 93–179.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514.

Halpern, J.L. and Neale, E.A. (1995). Neurospecific binding, internalization, and retrograde axonal transport. *Curr. Top. Microbiol. Immunol*. **195,** 221–241.

Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A. and Morris, A.J. (1997). Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. *J. Biol. Chem*. **272,** 3860–3868.

Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S. and Matsuda, M. (1992). Production, purification, and characterization of botunolysin, a thiol-activated hemolysin of *Clostridium botulinum. Infect. Immun*. **60,** 71–78.

Hassan, S.M., Jennekens, F.G., Wieneke, G. and Veldman, H. (1994). Calcitonin gene-related peptide-like immunoreactivity in botulinum toxin-paralysed rat muscles. *Neuromuscul. Disord*. **4,** 489–496.

Hatheway, C.L. (1993). *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin. In: *Clostridium botulinum*: *Ecology and Control in Foods*. (eds. A.H.W. Hauschild and K.L. Dodds), pp. 3–20. Marcel Dekker, Inc., New York.

Hayashi, T., McMahon, H., Yamashi, S., Binz, T., Hata, Y., Südhof, T.C. and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* **13,** 5051–5061.

Herreros, J., Lalli, G., Montecucco, C. and Schiavo, G. (1999). Pathophysiological properties of clostridial neurotoxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins*, vol. 2. (eds. J.E. Alouf and J.H. Freer), pp. 202–228. Academic Press, London.

Herreros, J., Lalli, G. and Schiavo, G. (2000). C-terminal half of tetanus toxin fragment C is sufficient for neuronal binding and interaction with a putative protein receptor. *Biochem. J.* **347,** 199–204.

Holds, J.B., Alderson, K., Fogg, S.G. and Anderson, R.L. (1990). Motor nerve sprouting in human orbicularis muscle after botulinum A injection. *Invest. Ophthalmol. Vis. Sci*. **31,** 964–967.

Hua, S.Y. and Charlton, M.P. (1999). Activity-dependent changes in partial VAMP complexes during neurotransmitter release. *Nat. Neurosci*. **2,** 1078–1083.

Hua, S.Y., Raciborska, D.A., Trimble, W.S. and Charlton, M.P. (1998). Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. *J. Neurophysiol*. **80,** 3233–3246.

Hua, Y. and Scheller, R.H. (2001). Three SNARE complexes cooperate to mediate membrane fusion. *Proc. Natl. Acad. Sci. USA* **98,** 8065–8070.

Humeau, Y., Doussau, F., Grant, N.J. and Poulain, B. (2000). How botulinum and tetanus neurotoxins block neurotransmitter. *Biochimie* **82,** 427–446.

Humeau, Y., Popoff, M.R., Kojima, H., Dousseau, F. and Poulain, B. (2002). Rac GTPase plays an essential role in exocytosis by controlling the fusion competence in release sites. *J. Neurosci*. **22,** 7968–7981.

Humeau, Y., Vitale, N., Chasserot-Golaz, S., Dupont, J.L., Du, G., Frohman, M.A., Bader, M.F. and Poulain, B. (2001). A role for phospholipase D1 in neurotransmitter release. *Proc. Natl. Acad. Sci. USA* **98,** 15300–15305.

Inoue, K., Fujinaga, Y., Honke, K., Yokota, K., Ikeda, T., Ohyama, T., Takeshi, K., Watanabe, T., Inoue, K. and Oguma, K. (1999). Characterization of hemagglutinin activity of *Clostridium botulinum* type C and D 16S toxins and one subcomponent of hemagglutinin (HA1). *Microbiol*ogy **145,** 2533–2542.

Inoue, K., Fujnaga, Y., Honke, K., Arimitsu, H., Mahmut, N., Sakaguchi, G., Ohyama, T., Watanabe, T., Inoue, K. and Oguma, K. (2001). *Clostridium botulinum* type A hemagglutinin positive progenitor toxin ($HA^+$-PTX) binds to oligosaccharides containing Galβ1-4GlcNAc through one subcomponent of hemagglutinin (HA1). *Microbiology* **147,** 811–819.

Inoue, K., Sobhany, M., Transue, T.R., Oguma, K., Pedersen, L.C. and Negishi, M. (2003). Structural analysis by X-ray crystallography and calorimetry of a hemagglutinin component (HA1) of the progenitor toxin from *Clostridium botulinum. Microbiology* **149,** 3361–3370.

Inserte, J., Najib, A., Pelliccioni, P., Gil, C. and Aguilera, J. (1999). Inhibition by tetanus toxin of sodium-dependent, high-affinity [3H]5-hydroxytryptamine uptake in rat synaptosomes. *Biochem. Pharmacol*. **57,** 111–120.

Ishida, H., Zhang, X., Erickson, K. and Ray, P. (2004). Botulinum toxin type A targets RhoB to inhibit lysophosphatidic acid-stimulated actin reorganization and acetylcholine release in nerve growth factor-treated PC12 cells. *J. Pharmacol. Exp. Ther*. **310,** 881–889.

Jahn, R., Lang, T. and Sudhof, T.C. (2003). Membrane fusion. *Cell* **112,** 519–533.

Jahn, R. and Sudhof, T.C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem*. **68,** 863–911.

Jett, M., Brinkley, W., Neill, R., Gemski, P. and Hunt, R. (1990). *Staphylococcus aureus* enterotoxin B challenge of monkeys: correlation of plasma levels of arachidonic acid cascade products with occurrence of illness. *Infect. Immun*. **58,** 3494–3499.

Ji, J., Tsuk, S., Salapatek, A.M., Huang, X., Chikvashvili, D., Pasyk, E.A., Kang, Y., Sheu, L., Tsushima, R., Diamant, N., Trimble, W.S., Lotan, I. and Gaisano, H.Y. (2002). The 25-kDa synaptosome-associated protein (SNAP-25) binds and inhibits delayed rectifier potassium channels in secretory cells. *J. Biol. Chem*. **277,** 20195–20204.

Johannes, L., Doussau, F., Clabecq, A., Henry, J.P., Darchen, F. and Poulain, B. (1996). Evidence for a functional link between Rab3 and the SNARE complex. *J. Cell. Sci*. **109** *(Pt 12)*, 2875–2884.

Juzans, P., Comella, J.X., Molgo, J., Faille, L. and Angaut-Petit, D. (1996a). Nerve terminal sprouting in botulinum type-A treated mouse *Levator auris Longus* muscle. *Neuromusc. Disord*. **6,** 177–185.

Juzans, P., Molgo, J., Faille, L. and Angaut-Petit, D. (1996b). Synaptotagmin II immunoreactivity in normal and botulinum type-A treated mouse motor nerve terminals. *Pflugers Arch*. **431,** R283–284.

Kalandakanond, S. and Coffield, J.A. (2001a). Cleavage of intracellular substrates of botulinum toxins A, C, and D in a mammalian target tissue. *J. Pharmacol. Exper. Ther*. **296,** 749–755.

Kalandakanond, S. and Coffield, J.A. (2001b). Cleavage of SNAP-25 by botulinum toxin type A requires receptor-mediated endocytosis, pH-dependent translocation, and zinc. *J. Pharmacol. Exp. Ther*. **296,** 980–986.

Keller, J.E. and Neale, E.A. (2001). The role of the synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. *J. Biol. Chem*. **276,** 13476–13482.

Keller, J.E., Neale, E.A., Oyler, G. and Adler, M. (1999). Persistence of botulinum neurotoxin action in cultured spinal cord cells. *FEBS Lett*. **456,** 137–142.

Koh, T.W. and Bellen, H.J. (2003). Synaptotagmin I, a $Ca^{2+}$ sensor for neurotransmitter release. *Trends Neurosci*. **26,** 413–422.

Koltgen, D., Ceballos-Baumann, A.O. and Franke, C. (1994). Botulinum toxin converts muscle acetylcholine receptors from adult to embryonic type. *Muscle Nerve* **17,** 779–784.

Kouguchi, H., Watanabe, T., Sagane, Y., Sunagawa, H. and Ohyama, T. (2002). *In vitro* reconstitution of the *Clostridium botulinum* type D progenitor toxin. *J. Biol. Chem*. **277,** 2650–2656.

Kowluru, A.,Li, G., Rabaglia, M.E., Segu, V.B., Hofmann, F., Aktories, K. and Metz, S.A. (1997). Evidence for differential roles of the Rho subfamily of GTP-binding proteins in glucose- and calcium-induced insulin secretion from pancreatic beta cells. *Biochem. Pharmacol*. **54,** 1097–1108.

Krakauer, T. and Stiles, B.G. (2003). Staphylococcal enterotoxins, toxic-shock syndrome toxin-1, and streptococcal pyrogenic exotoxins: some basic biology of bacterial superantigens. *Rec. Res. Dev. Infect. Immun*. **1,** 1–27.

Krauss, M., Kinuta, M., Wenk, M.R., De Camilli, P., Takei, K. and Haucke, V. (2003). ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type I gamma. *J. Cell. Biol*. **162,** 113–124.

Krieglstein, K.G., DasGupta, B.R. and Henschen, A.H. (1994). Covalent structure of botulinum neurotoxin A: location of sulfhydryl bridges and identification of C-termini of light and heavy chains. *J. Protein. Chem*. **13,** 49–57.

Krieglstein, K.G., Henschenn, A.H., Weller, U. and Habermann, E. (1991). Limited proteolysis of tetanus toxin. Relation to activity and identification of cleavage sites. *Eur. J. Biochem*. **202,** 41–51.

Kurazono, H., Mochida, S., Binz, T., Eisel, U., Quanz, M., Grebenstein, O., Wernars, K., Poulain, B., Tauc, L. and Niemann, H. (1992). Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A. *J. Biol. Chem*. **2667,** 14721–14729.

Lacy, D.B. and Stevens, R.C. (1999). Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol*. **291,** 1091–1104.

Lacy, D.B., Tepp, W., Cohen, A.C., Das Gupta, B.R. and Stevens, R.C. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nature Struct. Biol*. **5,** 898–902.

Lalli, G., Bohnert, S., Deinhardt, K., Verastegui, C. and Schiavo, G. (2003). The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol*. **11,** 431–437.

Lawrence, G.W., Foran, P., Mohammed, N., DasGupta, B.R. and Dolly, J.O. (1997). Importance of two adjacent C-terminal sequences of SNAP-25 in exocytosis from intact and permeabilized chromaffin cells revealed by inhibition with botulinum neurotoxins A and E. *Biochemistry* **36,** 3061–3067.

Li, L., Binz, T., Niemann, H. and Singh, B.R. (2000). Probing the mechanistic role of glutamate residue in the zinc-binding motif of type A botulinum neurotoxin light chain. *Biochemistry* **39,** 2399–2405.

Li, Q., Ho, C.S., Marinescu, V., Bhatti, H., Bokoch, G.M., Ernst, S.A., Holz, R.W. and Stuenkel, E.L. (2003). Facilitation of $Ca^{(2+)}$-dependent exocytosis by Rac1-GTPase in bovine chromaffin cells. *J. Physiol*. **550,** 431–445.

Linseman, D.A., Laessig, T., Meintzer, M.K., McClur, M., Barth, H., Aktories, K. and Heidenreich, K.A. (2001). An essential role for Rac/Cdc42 GTPases in cerebellar granule neuron survival. *J. Biol. Chem*. **276,** 23–31.

Lipsky, N.G., Drachman, D.B., Pestronk, A. and Shih, P.J. (1989). Neural regulation of mRNA for the alpha-subunit of acetylcholine receptors: role of neuromuscular transmission. *Exp. Neurol*. **105,** 171–176.

Low, P.A. (2002). Autonomic neuropathies. *Curr. Opin. Neurol*. **15,** 605–609.

Luo, J.Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L.A., Morris, A.J., Kahn, R.A. and Foster, D.A. (1998). Functional association between Arf and RalA in active phospholipase D complex. *Proc. Natl. Acad. Sci*. USA **95,** 3632–3637.

Luvisetto, S., Rossetto, O., Montecucco, C. and Pavone, F. (2003). Toxicity of botulinum neurotoxins in central nervous system of mice. *Toxicon* **41,** 475–481.

Mahmut, N., Inoue, K., Fujinaga, Y., Arimitsu, H., Sakaguchi, Y., Hughes, L., Hirst, R., Murphy, T., Tsuji, T., Watanabe, T., Ohyama, T., Karasawa, T., Nakamura, S., Yokota, K. and Oguma, K. (2002a). Mucosal immunization with *Clostridium botulinum* type C 16S toxoid and its non-toxic component. *J. Med. Microbiol*. **51,** 813–820.

Mahmut, N., Inoue, K., Fujinaga, Y., Hughes, L., Arimitsu, H., Sakaguchi, G., Ohtsuka, A., Murakami, T., Yokota, K. and Oguma, K. (2002b). Characterization of monoclonal antibodies against hemagglutinin associated with *Clostridium botulinum* type C neurotoxin. *J. Med. Microbiol*. **51,** 286–294.

Maksymowych, A.B., Rienhard, M., Malizio, C.J., Goodnough, M.C., Johnson, E.A. and Simpson, L.L. (1999). Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. *Infect. Immun*. **67,** 4708–4712.

Maksymowych, A.B. and Simpson, L.L. (1998). Binding and transcytosis of botulinum neurotoxin by polarized human carcinoma cells. *J. Biol. Chem*. **273,** 21950–21957.

Manning, K.A., Erichsen, J.T. and Evinger, C. (1990). Retrograde transneuronal transport properties of fragment C of tetanus toxin. *Neuroscience* **34,** 251–263.

Margittai, M., Otto, H. and Jahn, R. (1999). A stable interaction between syntaxin 1a and synaptobrevin 2 mediated by their transmembrane domains. *FEBS Lett*. **446,** 40–44.

Marxen, P. and Bigalke, H. (1991). Tetanus and botulinum A toxins inhibit stimulated F-actin rearrangement in chromaffin cells. *Neuroreport* **2,** 33–36.

Maskos, U., Kissa, K., St Cloment, C. and Brulet, P. (2002). Retrograde trans-synaptic transfer of green fluorescent protein allows the genetic mapping of neuronal circuits in transgenic mice. *Proc. Natl. Acad. Sci. USA* **99,** 10120–10125.

McMahon, H.T., Foran, P., Dolly, J.O., Verhage, M., Wiegant, V.M. and Nicholls, D.G. (1992). Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *J. Biol. Chem*. **267,** 21338–21343.

Meng, X., Karasawa, T., Zou, K., Kuang, X., Wang, X., Lu, C., Wang, C., Yamakawa, K. and Nakamura, S. (1997). Characterization of a neurotoxigenic *Clostridium butyricum* strain isolated from the food implicated in an outbreak of food-borne type E botulism. *J. Clin. Microbiol*. **35,** 2160–2162.

Meunier, F.A., Lisk, G., Sesardic, D. and Dolly, J.O. (2003). Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: the extent and duration are dictated by the sites of SNAP-25 truncation. *Mol. Cell. Neurosci*. **22,** 454–466.

Meunier, F.A., Schiavo, G. and Molgo, J. (2002). Botulinum neurotoxins: from paralysis to recovery of functional neuromuscular transmission. *J. Physiol*. **96,** 105–113.

Meyer, D.K., Olenik, C., Hofmann, F., Barth, H., Leemhuis, J., Brunig, I., Aktories, K. and Norenberg, W. (2000). Regulation of somatodendritic GABAA receptor channels in rat hippocampal neurons: evidence for a role of the small GTPase Rac1. *J. Neurosci*. **20,** 6743–6751.

Miana-Mena, F.J., Roux, S., Benichou, J.C., Osta, R. and Brulet, P. (2002). Neuronal activity-dependent membrane traffic at the neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **99,** 3234–3239.

Michaelevski, I., Chikvashvili, D., Tsuk, S., Singer-Lahat, D., Kang, Y., Linial, M., Gaisano, H.Y., Fili, O. and Lotan, I. (2003). Direct interaction of target SNAREs with the Kv2.1 channel. Modal regulation of channel activation and inactivation gating. *J. Biol. Chem*. **278,** 34320–34330.

Miyamoto, O., Minami, J., Toyoshima, T., Nakamura, T., Masada, T., Nagao, S., Negi, T., Itano, T. and Okabe, A. (1998). Neurotoxicity

of *Clostridium perfringens* epsilon-toxin for the rat hippocampus via glutamanergic system. *Infect. Immun*. **66,** 2501–2508.

Miyamoto, O., Sumitami, K., Nakamura, T., Yamagani, S., Miyatal, S., Itano, T., Negi, T. and Okabe, A. (2000). *Clostridium perfringens* epsilon toxin causes excessive release of glutamate in the mouse hippocampus. *FEMS Microbiol. Lett*. **189,** 109–113.

Miyata, S., Matsushita, O., Minami, J., Katayama, S., Shimamoto, S. and Okabe, A. (2001). Cleavage of C-terminal peptide is essential for heptamerization of *Clostridium perfringens* ε-toxin in the synaptosomal membrane. *J. Biol. Chem*. **276,** 13778–13783.

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S. and Okabe, A. (2002). *Clostridium perfringens* ε-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. *J. Biol. Chem*. **277,** 39463–39468.

Molgo, J., Comella, J.X., Angaut-Petit, D., Pecot-Dechavassine, M., Tabti, N., Faille, L., Mallart, A. and Thesleff, S. (1990). Presynaptic actions of botulinal neurotoxins at vertebrate neuromuscular junctions. *J. Physiol. Paris* **84,** 152–166.

Molgo, J., Siegel, L.S., Tabti, N. and Thesleff, S. (1989). A study of synchronization of quantal transmitter release from mammalian motor endings by the use of botulinal toxins type A and D. *J. Physiol*. **411,** 195–205.

Moreno-Lopez, B., de la Cruz, R.R., Pastor, A.M. and Delgado-Garcia, J.M. (1997). Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: alterations of the discharge pattern. *Neuroscience* **81,** 437–455.

Morris, J.L., Jobling, P. and Gibbins, I.L. (2001). Differential inhibition by botulinum neurotoxin A of cotransmitters released from autonomic vasodilator neurons. *Am. J. Physiol. Heart Circ. Physiol*. **281,** H2124–2132.

Murray, H.J. and O'Connor, J.J. (2004). A role for monomeric G-proteins in synaptic plasticity in the rat dentate gyrus *in vitro*. *Brain Res*. **1000,** 85–91.

Murthy, V.N. and De Camilli, P. (2003). Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci*. **26,** 701–728.

Mutoh, S., Kouguchi, H., Sagane, Y., Suzuki, T., Hasegawa, K., Watanabe, T. and Ohyama, T. (2003). Complete subunit structure of the *Clostridium botulinum* type D complex via intermediate assembly with nontoxic components. *Biochemistry*. **42,** 10991–10997.

Nagahama, M. and Sakurai, J. (1991). Distribution of labeled *Clostridium perfringens* epsilon toxin in mice. *Toxicon* **29,** 211–217.

Nagahama, M. and Sakurai, J. (1992). High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. *Infect. Immun*. **60,** 1237–1240.

Najib, A., Peliccioni, P., Gil, C. and Aguilera, J. (1999). Clostridium neurotoxins influence serotonin uptake and release differently in rat brain synaptosomes. *J. Neurochem*. **72,** 1991–1998.

Naumann, M. and Jost, W. (2004). Botulinum toxin treatment of secretory disorders. *Mov. Disord*. **19 Suppl 8,** S137–141.

Nevins, A.K. and Thurmond, D.C. (2005). A direct interaction between Cdc42 and vesicle-associated membrane protein 2 regulates SNARE-dependent insulin exocytosis. *J. Biol. Chem*. **280,** 1944–1952.

New, H.V. and Mudge, A.W. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* **323,** 809–811.

Nishikawa, A., Uotsu, N., Arimitsu, H., Lee, J.C., Miura, Y., Fujinaga, Y., Nakada, H., Watanabe, T., Ohyama, T., Sakano, Y. and Oguma, K. (2004). The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells. *Biochem. Biophys. Res. Commun*. **319,** 327–333.

O'Connor, V., Heuss, C., De Bello, W.M., Dresbach, T., Charlton, M.P., Hunt, J.H., Pellegrini, L.L., Hodel, A., Burger, M.M., Betz, H., Augustine, G.J. and Schafer, T. (1997). Disruption of syntaxin-mediated protein interactions blocks neurotransmitter secretion. *Proc. Natl. Acad. Sci. USA* **94,** 12186–12191.

O'Sullivan, G.A., Mohammed, N., Foran, P.G., Lawrence, G.W. and Dolly, J.O. (1999). Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25. *J. Biol. Chem*. **274,** 36897–36904.

Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K. and Inoue, K. (1999). Structure and function of *Clostridium botulinum* progenitor toxin. *J. Toxicol*. **18,** 17–34.

Ohishi, I. and Sakaguchi, G. (1980). Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. *Infect. Immun*. **28,** 303–309.

Ohishi, I., Sugii, S. and Sakaguchi, G. (1977). Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. *Infect. Immun*. **16,** 107–109.

Osborne, S.L., Meunier, F.A. and Schiavo, G. (2001). Phosphoinositides as key regulators of synaptic function. *Neuron* **32,** 9–12.

Park, J.B. and Simpson, L.L. (2003). Inhalation poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. *Infect. Immun*. **71,** 1147–1154.

Pastor, A.M., Moreno-Lopez, B., De La Cruz, R.R. and Delgado-Garcia, J.M. (1997). Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: ultrastructural and synaptic alterations. *Neuroscience* **81,** 457–478.

Petersen, O.H. (2003). Localization and regulation of $Ca^{2+}$ entry and exit pathways in exocrine gland cells. *Cell Calcium* **33,** 337–344.

Petit, L., Gibert, M., Gillet, D., Laurent-Winter, C., Boquet, P. and Popoff, M.R. (1997). *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. *J. Bacteriol*. **179,** 6480–6487.

Petit, L., Gibert, M., Gourch, A., Bens, M., Vandewalle, A. and Popoff, M.R. (2003). *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. *Cell. Microbiol*. **5,** 155–164.

Petit, L., Maier, E., Gibert, M., Popoff, M.R. and Benz, R. (2001). *Clostridium perfringens* epsilon-toxin induces a rapid change in cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J. Biol. Chem*. **276,** 15736–15740.

Poirier, M.A., Xiao, W., Macosko, J.C., Chan, C., Shin, Y.K. and Bennett, M.K. (1998). The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Biol*. **5,** 765–769.

Polzin, A., Shipitsin, M., Goi, T., Feig, L.A. and Turner, T.J. (2002). Ral-GTPase influences the regulation of the readily releasable pool of synaptic vesicles. *Mol. Cell. Biol*. **22,** 1714–1722.

Popoff, M.R. and Marvaud, J.C. (1999). Structural and genomic features of clostridial neurotoxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins*, vol. 2 (eds. J.E. Alouf and J.H. Freer), pp. 174–201. Academic Press, London.

Pothoulakis, C., Castagliuolo, I. and LaMont, J.T. (1998). Nerves and intestinal mast cells modulate responses to enterotoxins. *News Physiol. Sci*. **13,** 58–63.

Pothoulakis, C. and Lamont, J.T. (2001). Microbes and microbial toxins: paradigms for microbial-mucosa interactions II. The integrated response of the intestine to *Clostridium difficile* toxins. *Am. J. Physiol. Gastrointest. Liver Physiol*. **280,** G178–G183.

Poulain, B., de Paiva, A., Deloye, F., Doussau, F., Tauc, L., Weller, U. and Dolly, J.O. (1996). Differences in the multiple step process of inhibition of neurotransmitter release induced by tetanus toxin and botulinum neurotoxins type A and B at *Aplysia* synapses. *Neurosci*. **70,** 567–576.

Poulain, B., Bader, M.F. and Molgo, J. (2000). Physiological approaches for studying clostridial neurotoxin mechanisms. *Meth. Molec. Biol*. **145,** 259–286.

Prepens, U., Just, I., von Eichel-Streiber, C. and Aktories, K. (1996). Inhibition of Fc epsilon-RI-mediated activation of rat basophilic leukemia cells by *Clostridium difficile* toxin B (monoglucosyltransferase). *J. Biol. Chem*. **271,** 7324–7329.

Presek, P., Jessen, S., Dreyer, F., Jarvie, P.E., Findik, D. and Dunkley, P.R. (1992). Tetanus toxin inhibits depolarization-stimulated protein phosphorylation in rat cortical synaptosomes: effect on synapsin I phosphorylation and translocation. *J. Neurochem*. **59,** 1336–1343.

Puffer, E.B., Lomneth, R.B., Sarkar, H.K. and Singh, B.R. (2001). Differential roles of developmentally distinct SNAP-25 isoforms in the neurotransmitter release process. *Biochemistry* **40,** 9374–9378.

Purkiss, J., Welch, M., Doward, S. and Foster, K. (2000). Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. *Biochem. Pharmacol*. **59,** 1403–1406.

Quetglas, S., Leveque, C., Miquelis, R., Sato, K. and Seagar, M. (2000). $Ca^{2+}$-dependent regulation of synaptic SNARE complex assembly via a calmodulin- and phospholipid-binding domain of synaptobrevin. *Proc. Natl. Acad. Sci. USA* **97,** 9695–9700.

Quinn, C.P. and Minton, N.P. (2001). Clostridial neurotoxins. In: *Clostridia*, (eds. H. Bahl and P. Dürre), pp. 211–250. Willey-VCH, Veinheim.

Raciborska, D.A., Trimble, W.S. and Charlton, M.P. (1998). Presynaptic protein interactions *in vivo:* evidence from botulinum A, C, D, and E action at frog neuromuscular junction. *Eur. J. Neurosci*. **10,** 2617–2628.

Rettig, J. and Neher, E. (2002). Emerging roles of presynaptic proteins in $Ca^{++}$-triggered exocytosis. *Science* **298,** 781–785.

Reisinger, C., Yelamanchili, S.V., Hinz, B., Mitter, D., Becher, A., Bigalke, H. and Ahnert-Hilger, G. (2004). The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis. *J. Neurochem*. **90,** 1–8.

Rigoni, M., Caccin, P., Johnson, E.A., Montecucco, C. and Rossetto, O. (2001). Site-directed mutagenesis identifies active-site residues of the light chain of botulinum neurotoxin A. *Biochem. Biophys. Res. Commun*. **288,** 1231–1237.

Rolfe, V.E. and Levin, R.J. (1999). Vagotomy inhibits the jejunal fluid secretion activated by luminal ileal *Escherichia coli* STa in the rat *in vivo. Gut* **44,** 615–619.

Rosado, J.A., Redondo, P.C., Salido, G.M., Sage, S.O. and Pariente, J.A. (2005). Cleavage of SNAP-25 and VAMP-2 impairs store-operated $Ca^{2+}$ entry in mouse pancreatic acinar cells. *Am. J. Physiol. Cell Physiol*. **288,** C214–221.

Rosales, R.L., Arimura, K., Takenaga, S. and Osame, M. (1996). Extrafusal and intrafusal muscle effects in experimental botulinum toxin-A injection. *Muscle Nerve* **19,** 488–496.

Rosato Siri, M.D. and Uchitel, O.D. (1999). Calcium channels coupled to neurotransmitter release at neonatal rat neuromuscular junctions. *J. Physiol*. **514** (Pt 2), 533–540.

Rossetto, O., Caccin, P., Rigoni, M., Tonello, F., Bortoletto, N., Stevens, R.C. and Montecucco, C. (2001). Active-site mutagenesis of tetanus neurotoxin implicates Tyr-375 and Glu-271 in metalloproteolytic activity. *Toxicon* **39,** 1151–1159.

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloye, F., Lozzi, L. and Shone, C.C. (1994). SNARE motif and neurotoxins. *Nature* **372,** 415–416.

Rossi, S.G., Dickerson, I.M. and Rotundo, R.L. (2003). Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression. *J. Biol. Chem*. **278,** 24994–25000.

Rummel, A., Bade, S., Alves, J., Bigalke, H. and Binz, T. (2003). Two carbohydrate binding sites in the $H_{cc}$-domain of tetanus neurotoxin are required for toxicity. *J. Mol. Biol*. **326,** 835–847.

Rummel, A., Mahrhold, S., Bigalke, H. and Binz, T. (2004). The $H_{cc}$-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. *Mol. Microbiol*. **51,** 631–643.

Sadoul, K., Berger, A., Niemann, H., Regazzi, R., Catsicas, S. and Halban, P.A. (1997). SNAP-25 can self-associate to form a disulfide-linked complex. *J. Biol. Chem*. **378,** 1171–1176.

Sagane, Y., Kouguchi, H., Watanabe, T., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K. and Ohyama, T. (2001). Role of C-terminal region of HA-33 component of botulinum toxin in hemagglutination. *Biochem. Biophys. Res. Commun*. **288,** 650–657.

Sagane, Y., Watanabe, T., Kouguchi, H., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K. and Ohyama, T. (1999). Dichain structure of botulinum neurotoxin: identification of cleavage sites in types C, D, and F neurotoxin molecules. *J. Protein Chem*. **18,** 885–892.

Sagane, Y., Watanabe, T., Kouguchi, H., Sunagawa, H., Inoue, K., Fujinaga, Y., Oguma, K. and Ohyama, T. (2000). Characterization of nicking of the nontoxic-nonhemagglutinin components of *Clostridium botulinum* types C and D progenitor toxin. *J. Protein Chem*. **19,** 575–581.

Sala, C., Andreose, J.S., Fumagalli, G. and Lomo, T. (1995). Calcitonin gene-related peptide: possible role in formation and maintenance of neuromuscular junctions. *J. Neurosci*. **15,** 520–528.

Salem, N., Faundez, V., Horng, J.T. and Kelly, R.B. (1998). A v-SNARE participates in synaptic vesicle formation mediated by the AP3 adaptor complex. *Nat. Neurosci*. **1,** 551–556.

Sanes, J.R. and Lichtman, J.W. (1999). Can molecules explain long-term potentiation? *Nat. Neurosci*. **2,** 597–604.

Santafe, M.M., Urbano, F.J., Lanuza, M.A. and Uchitel, O.D. (2000). Multiple types of calcium channels mediate transmitter release during functional recovery of botulinum toxin type A-poisoned mouse motor nerve terminals. *Neuroscience* **95,** 227–234.

Santini, M., Fabri, S., Sagnelli, P., Manfredi, M. and Francia, A. (1999). Botulism: a case associated with pyramidal signs. *Eur. J. Neurol*. **6,** 91–93.

Schiavo, G., Matteoli, M. and Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. *Physiol. Rev*. **80,** 717–766.

Schuette, C.G., Hatsuzawa, K., Margittai, M., Stein, A., Riedel, D., Kuster, P., Konig, M., Seidel, C. and Jahn, R. (2004). Determinants of liposome fusion mediated by synaptic SNARE proteins. *Proc. Natl. Acad. Sci. USA* **101,** 2858–2863.

Segev, N. (2001). Ypt/rab gtpases: regulators of protein trafficking. *Sci STKE* **100,** RE11.

Shapiro, R.E., Spech, C.D., Collins, B.E., Woods, A.S., Cotter, R.J. and Schnaar, R.L. (1997). Identification of a ganglioside recognition domain of tetanus toxin using a novel ganglioside photoaffinity ligand. *J. Biol. Chem*. **272,** 30380–30386.

Sharma, S.K., Fu, F.N. and Singh, B.R. (1999). Molecular properties of a hemagglutinin purified from type A *Clostridium botulinum. J. Protein Chem*. **18,** 29–38.

Sharma, S.K., Ramzan, M.A. and Singh, B.R. (2003). Separation of the components of type A botulinum neurotoxin complex by electrophoresis. *Toxicon* **41,** 321–331.

Sharma, S.K. and Singh, B.R. (2004). Enhancement of the endopeptidase activity of purified botulinum neurotoxins A and E by an isolated component of the native neurotoxin associated proteins. *Biochemistry* **43,** 4791–4798.

Short, B. and Barr, F.A. (2002). Membrane traffic: exocyst III—makes a family. *Curr. Biol*. **12,** R18–20.

Simpson, L.L. (1989). Botulinum neurotoxin and tetanus toxin. Academic Press: San Diego.

Simpson, L.L. (2004). Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol*. **44,** 167–193.

Son, Y.J. and Thompson, W.J. (1995). Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. *Neuron* **14,** 133–141.

Sorensen, J.B., Nagy, G., Varoqueaux, F., Nehring, R.B., Brose, N., Wilson, M.C. and Neher, E. (2003). Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell* **114,** 75–86.

Stanley, E.F. and Mirotznik, R.R. (1997). Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. *Nature* **385,** 340–343.

Stanley, E.F., Reese, T.S. and Wang, G.Z. (2003). Molecular scaffold reorganization at the transmitter release site with vesicle exocytosis or botulinum toxin C1. *Eur. J. Neurosci*. **18,** 2403–2407.

Suen, J.C., Hatheway, C.L., Steigerwalt, A.G. and Brenner, D.J. (1988). *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* toxin type G and some nontoxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme. Int. J. Syst. Bacteriol*. **38,** 375–381.

Sutton, R.B., Fasshauer, D., Jahn, R. and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 angstrom resolution. *Nature* **395,** 347–353.

Swaminathan, S. and Eswaramoorthy, S. (2000). Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nature Struct. Biol*. **7,** 693–699.

Szule, J.A. and Coorssen, J.R. (2003). Revisiting the role of SNAREs in exocytosis and membrane fusion. *Biochim. Biophys. Acta*. **1641,** 121–135.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Schelleer, R.H. and Rothmann, J.E. (1993). A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75,** 409–418.

Tacket, C.O. and Rogawski, M.A. (1989). Botulism. In: *Botulinum Neurotoxin and Tetanus Toxin*. (ed. L.L. Simpson), pp. 351–378 Academic Press, San Diego.

Tarabal, O., Caldero, J., Ribera, J., Sorribas, A., Lopez, R., Molgo, J. and Esquerda, J.E. (1996). Regulation of motoneuronal calcitonin gene-related peptide (CGRP) during axonal growth and neuromuscular synaptic plasticity induced by botulinum toxin in rats. *Eur. J. Neurosci*. **8,** 829–836.

Tavallaie, M., Chenal, A., Gillet, D., Raffestin, S., Popoff, M.R. and Marvaud, J.C. (2004). Interaction between the two subdomains of the C-terminal part of the botulinum neurotoxin A is essential for the generation of protective antibodies. *FEBS Lett*. **572,** 299–306.

Thesleff, S. (1989). Botulinal neurotoxins as tools in studies of synaptic mechanisms. *Q. J. Exp. Physiol*. **74,** 1003–1017.

Thesleff, S., Molgo, J. and Tagerud, S. (1990). Trophic interrelations at the neuromuscular junction as revealed by the use of botulinal neurotoxins. *J. Physiol. (Paris)* **84,** 167–173.

Tian, W.H., Festoff, B.W., Blot, S., Diaz, J. and Hantai, D. (1995). Synaptic transmission blockade increases plasminogen activator activity in mouse skeletal muscle poisoned with botulinum toxin type A. *Synapse* **20,** 24–32.

Tiegs, G., Bang, R. and Neuhuber, W.L. (1999). Requirement of peptidergic sensory innervation for disease activity in murine models of immune hepatitis and protection by beta-adrenergic stimulation. *J. Neuroimmunol*. **96,** 131–143.

Tonge, D.A. (1974). Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetylcholine in slow and fast skeletal muscle of the mouse. *J. Physiol. (London)* **241,** 127–139.

Triller, A. and Choquet, D. (2003). Synaptic structure and diffusion dynamics of synaptic receptors. *Biol. Cell* **95,** 465–476.

Tscheng, D.Z. (2002). Sialorrhea—therapeutic drug options. *Ann. Pharmacother*. **36,** 1785–1790.

Tsuk, S., Michaelevski, I., Bentley, G.N., Joho, R.H., Chikvashvili, D. and Lotan, I. (2005). Kv2.1 channel activation and inactivation is influenced by physical interactions of both syntaxin 1A and the t-SNARE complex with the C-terminus of the channel. *Mol. Pharmacol*. **67,** 480–488.

Tucker, W.C., Weber, T. and Chapman, E.R. (2004). Reconstitution of $Ca^{2+}$-regulated membrane fusion by synaptotagmin and SNAREs. *Science* **304,** 435–438.

Tunkel, A.R. and Pradhan, S.K. (2002). Central nervous system infections in injection drug users. *Infect. Dis. Clin. North Am*. **16,** 589–605.

Turton, K., Chaddock, J.A. and Acharya, K.R. (2002). Botulinum and tetanus neurotoxins: structure, function, and therapeutic utility. *Trends Biochem. Sci*. **27,** 552–558.

Umland, T.C., Wingert, L.M., Swaminathan, S., Furey, W.F., Schmidt, J.J. and Sax, M. (1997). The structure of the receptor binding fragment $H_c$ of tetanus neurotoxin. *Nature Struct. Biol*. **4,** 788–792.

Van der Kloot, W. and Molgó, J. (1994). Quantal acetylcholine release at the vertebrate neuromuscular junction. *Physiol. Rev*. **74,** 899–991.

Verderio, C., Coco, S., Rossetto, O., Montecucco, C. and Matteoli, M. (1999). Internalization and proteolytic action of botulinum toxins in CNS neurons and astrocytes. *J. Neurochem*. **73,** 372–379.

Verderio, C., Pozzi, D., Pravettoni, E., Inverardi, F., Schenk, U., Coco, S., Proux-Gillardeaux, V., Galli, T., Rossetto, O., Frassoni, C. and Matteoli, M. (2004). SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron* **41,** 599–610.

Vitale, N., Caumont, A.S., Chasserot-Golaz, S., Du, G., Wu, S., Sciorra, V.A., Morris, A.J., Frohman, M.A. and Bader, M.F. (2001). Phospholipase D1: a key factor for the exocytic machinery in neuroendocrine cells. *EMBO J*. **20,** 2424–2434.

Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M.A. and Bader, M.F. (2002). Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. *J. Cell Biol*. **159,** 79–89.

Wang, X., Wang, B.R., Zhang, X.J., Duan, X.L., Guo, X. and Ju, G. (2004). Fos expression in the rat brain after intraperitoneal injection of *Staphylococcus* enterotoxin B and the effect of vagotomy. *Neurochem. Res*. **29,** 1667–1674.

Washbourne, P., Pellizzari, R., Baldini, G., Wilson, M.C. and Montecucco, C. (1997). Botulinum neurotoxin types A and E require the SNARE motif in SNAP-25 for proteolysis. *FEBS Lett*. **418,** 1–5.

Wei, S., Xu, T., Ashery, U., Kollewe, A., Matti, U., Antonin, W., Rettig, J. and Neher, E. (2000). Exocytotic mechanism studied by truncated and zero layer mutants of the C-terminus of SNAP-25. *Embo J*. **19,** 1279–1289.

Welch, M.J., Purkiss, J.R. and Foster, K.A. (2000). Sensitivity of embryonic rat dorsal root ganglia neurons to *Clostridium botulinum* neurotoxins. *Toxicon* **38,** 245–258.

Wellhöner, H.H. (1992). Tetanus and botulinum neurotoxins. In: *Selective Neurotoxicity*, vol. 102 (eds. H. Herken and F. Hucho) pp. 357–417. Springer-Verlag, Berlin.

Whelchel, D.D., Brehmer, T.M., Brooks, P.M., Darragh, N. and Coffield, J.A. (2004). Molecular targets of botulinum toxin at the mammalian neuromuscular junction. *Mov. Disord*. **19** *Suppl 8*, S7–S16.

Wiser, O., Trus, M., Hernandez, A., Renstrom, E., Barg, S., Rorsman, P. and Atlas, D. (1999). The voltage sensitive Lc-type $Ca^{2+}$ channel is functionally coupled to the exocytotic machinery. *Proc. Natl. Acad. Sci. USA* **96,** 248–253.

Witzemann, V., Brenner, H.R. and Sakmann, B. (1991). Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol*. **114,** 125–141.

Xia, Y., Hu, H.Z., Liu, S., Pothoulakis, C. and Wood, J.D. (2000). *Clostridium difficile* toxin A excites enteric neurones and suppresses sympathetic neurotransmision in the guinea pig. *Gut* **46,** 481–486.

Xu, T., Binz, T., Niemann, H. and Neher, E. (1998). Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity. *Nat. Neurosci*. **1,** 192–200.

Yang, J.F., Cao, G., Koirala, S., Reddy, L.V. and Ko, C.P. (2001). Schwann cells express active agrin and enhance aggregation of acetylcholine receptors on muscle fibers. *J. Neurosci*. **21,** 9572–9584.

Yersin, A., Hirling, H., Steiner, P., Magnin, S., Regazzi, R., Huni, B., Huguenot, P., De los Rios, P., Dietler, G., Catsicas, S. and Kasas, S. (2003). Interactions between synaptic vesicle fusion proteins explored by atomic force microscopy. *Proc. Natl. Acad. Sci. USA* **100,** 8736–8741.

Zhang, X., Kim-Miller, M.J., Fukuda, M., Kowalchyk, J.A. and Martin, T.F. (2002). $Ca^{2+}$-dependent synaptotagmin binding to SNAP-25 is essential for $Ca^{2+}$-triggered exocytosis. *Neuron* **34,** 599–611.

Zhu, C., Ghabriel, M.N., Blumbergs, P.C., Reilly, P.L., Manavis, J., Youssef, J., Hatami, S. and Finnie, J.W. (2001). *Clostridium perfringens* prototoxin-induced alteration of endothelial barrier antigen (EBA) immunoreactivity at the blood brain barrier (BBB). *Exp. Neurol*. **169,** 72–82.

CHAPTER

# 20

# Uptake and transport of clostridium neurotoxins

*Stephanie Bohnert, Katrin Deinhardt, Sara Salinas, and Giampietro Schiavo*

## INTRODUCTION

Over the last 100 years the causative agents of tetanus and botulism and the molecules responsible for their symptoms, the clostridium neurotoxins (CNT), have been studied intensely. Despite their recent fame, the clinical effects of CNT have been known since the beginning of medical literature. Hippocrates described for the first time the symptoms of a sailor affected by hypercontraction of skeletal muscle, which he termed $\tau\varepsilon\tau\alpha\nu o\sigma$ for tension. Tetanus was considered a neurological disease until the end of the nineteenth century, when a potent neurotoxin produced by *Clostridium tetani*, named Tetanus Neurotoxin (TeNT), was identified as the sole causative agent of the spastic paralysis characteristic of tetanus, which is often fatal due to cardiovascular or respiratory failure. The same period also saw the first description of botulism, a general muscle weakness, followed by the isolation of *Clostridium botulinum* and its neurotoxins (Botulinum Neurotoxins, BoNTs).

The isolation of *C. tetani* and *C. botulinum* toxigenic strains in the late 1980s and studies on their intracellular activity and their synaptic targets in the 1990s have revealed important insights into a complex protein machinery able to recognize neurons, enter the cytosol, and modify synaptic function. The description of CNT and the study of their molecular mechanism of action are central to several disciplines, including microbiology, pharmacology, physiology, biochemistry, and medicine. Furthermore, these neurotoxins have been used as molecular tools to dissect regulated secretion and intracellular trafficking in a variety of cells. Recently, BoNTs have been used increasingly in human therapy, mainly for pathologies caused by synaptic hyperactivity and as cosmetic agents.

## CLOSTRIDIUM NEUROTOXINS

CNT are the most toxic substances known, with mouse Lethal Dose 50% ($LD_{50}$) ranging between 0.1 and 1 ng $kg^{-1}$ of body weight. Different animals show a great range of sensitivity to TeNT and BoNTs. Mammals, except rats, are among the most receptive species, whereas birds, reptiles, and amphibians are resistant to CNT to different extents (Gill, 1982). The time of onset of paralysis after CNT intoxication is dependent on the species, dose, and route of application. However, a delay ranging from several hours up to days is always present between infection with CNT and manifestation of the earliest clinical symptoms.

After entering the general circulation, CNT bind to the presynaptic membrane of the neuromuscular junction (NMJ) (Figure 20.1). This binding is highly specific and at high affinity, contributing together with the enzymatic activity of CNT to the very low $LD_{50}$ values and the limited spreading around the site of injection observed *in vivo*. Following binding, CNT are internalized into the neuron. BoNTs remain at the NMJ and block the release of the excitatory neurotransmitter acetylcholine (ACh), causing a flaccid paralysis (Figure 20.1b). In contrast, TeNT is retrogradely transported to

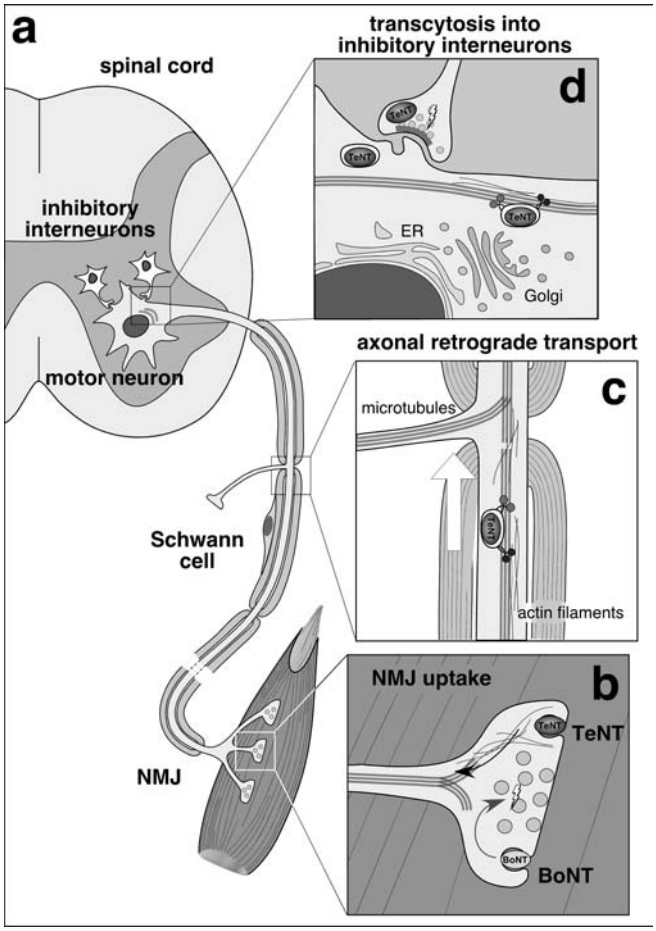



**FIGURE 20.1** Distinct trafficking of TeNT and BoNT *in vivo*. (**a**) Scheme of the anatomical connections between skeletal muscles, spinal cord, motor neurons (MN), and their afferent cells. MN control skeletal muscles with a specialized synapse termed *neuromuscular junction* (NMJ). The MN axon, which is wrapped by a myelin sheet produced by Schwann cells, can reach a length of a meter or more in large mammals. The MN soma is located in the spinal cord, where it forms contacts with adjacent interneurons. (**b**) NMJ uptake. After entering the general circulation, CNT reach the NMJ where they bind to the presynaptic terminal and are internalized. BoNTs remain at the NMJ, where they cleave SNARE proteins, thereby blocking the fusion of synaptic vesicles (SV). The consequent inhibition in ACh release causes a flaccid paralysis. In contrast, upon endocytosis TeNT enters specialized transport carriers directed towards the MN soma located in the spinal cord, where it accumulates in the ventral horn of the gray matter. (**c**) Axonal retrograde transport. Crucial elements of the axonal transport machinery are motor proteins that use ATP to perform mechanical work. Microtubule-based motors (cytoplasmic dynein and kinesins) play a major role in axonal transport and are MT-based. However, actin-dependent motors are also relevant for this process, indicating a functional connection between MT and actin-based transport. (**d**) Transcytosis into inhibitory interneurons. Once in the spinal cord, TeNT is released into the extracellular medium and internalized into adjacent inhibitory interneurons, where it cleaves VAMP, thereby blocking inhibitory neurotransmission. This impairs the balance between inhibitory and excitatory afferents on the MN, which ensures coordinated muscle contraction and causes spastic paralysis.

the motor neuron (MN) soma located in the spinal cord, where it accumulates in the ventral horn of the gray matter (Figure 20.1c). Once in the spinal cord, TeNT is released into the extracellular medium and internalized into adjacent inhibitory interneurons, where it blocks the release of inhibitory neurotransmitters (Figure 20.1d). This impairs the balance between inhibitory and excitatory afferents on the MN, which ensures coordinated muscle contraction and causes spastic paralysis. Despite their different clinical symptoms and intracellular targeting, the mode of action of TeNT and BoNTs is therefore similar. All CNT block neurotransmitter release and this inhibition is due to specific cleavage of soluble NSF attachment protein receptors (SNARE) involved in neuroexocytosis.

TeNT can bind sensory as well as adrenergic neurons, which exhibit a similar retrograde uptake. Although excitatory synapses appear not to be compromised in the early stages of the disease, they may be inhibited at later periods of TeNT intoxication. This preference for inhibitory versus excitatory synapses is maintained when TeNT is applied directly into the central nervous system (CNS) and may underlie the neurodegenerative and epileptogenic effects of TeNT, which result from unopposed release of glutamate from excitatory central synapses.

## STRUCTURE-FUNCTION RELATIONSHIP

As expected from their similar mechanism of action, toxins from the CNT family share a high degree of sequence and structural homology. CNT are produced as a single-chain inactive polypeptide of 150 kDa, which will be cleaved by endogenous or exogenous proteases (DasGupta, 1994; Weller *et al*., 1989) (Figure 20.2). This specific cleavage produces two chains, a 100 kDa heavy chain (H chain) and a 50 kDa light chain (L chain). These chains remain associated via non-covalent protein-protein interactions and a conserved inter-chain disulphide bond, which is essential for neurotoxicity (de Paiva *et al*., 1993; Schiavo *et al*., 1990a). The H chain can be further subdivided into two different fragments, the 50 kDa amino-terminal part ($H_N$) and the 50 kDa carboxy-terminal part ($H_C$) (Bizzini *et al*., 1977; Helting and Zwisler, 1977; Neubauer and Helting, 1981) (Figure 20.2b).

The existence of these three functional domains was confirmed by the crystallization and structural determination of BoNT/A (Lacy *et al*., 1998; Stevens *et al*., 1991) (Figure 20.2a), BoNT/B (Swaminathan and Eswaramoorthy, 2000), and the L chain and $H_C$ domain of TeNT (Umland *et al*., 1997; Rao *et al*., 2005) (Figure 20.2c). At a 3.3

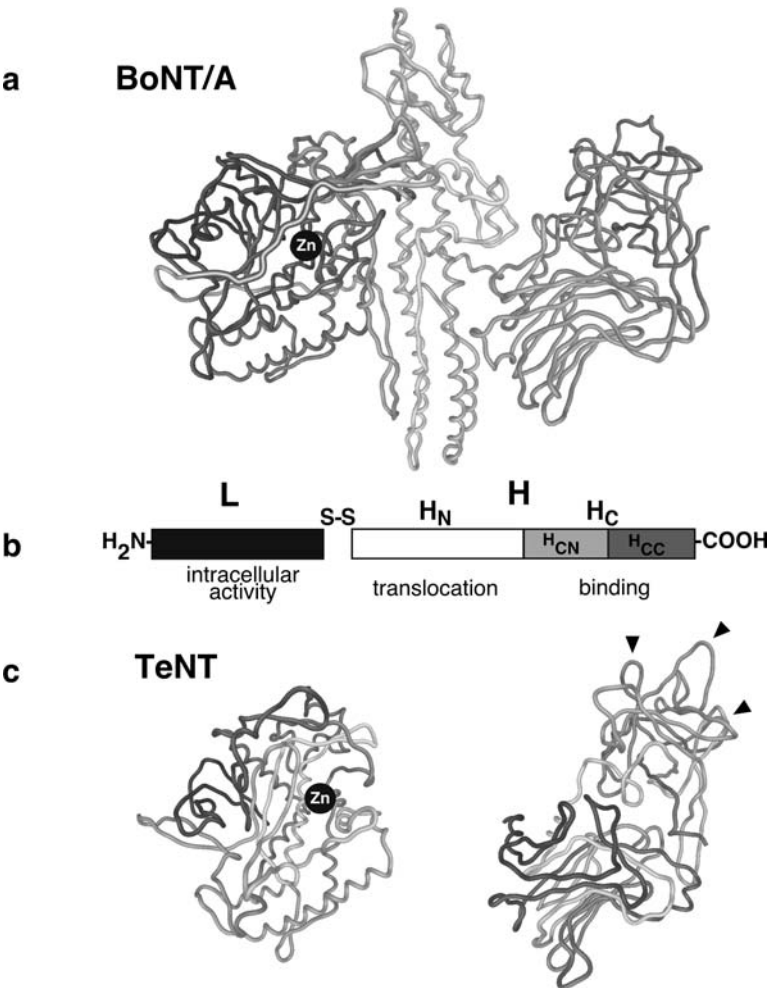



**FIGURE 20.2** Structure of CNT (**a**) Crystal structure of BoNT/A. The binding domain is highlighted in gray, the translocation domain in light gray and the catalytic domain, coordinating a zinc atom (large dark sphere), in dark gray. The catalytic domain is formed by a mixture of β-strands and α-helices. The translocation domain consists of two long α-helices and a long belt wrapping around the catalytic domain, here highlighted in white. The amino terminal part of $H_C$ displays two seven-stranded β-sheets ($H_{CN}$), whereas the carboxy-terminus adopts a modified β-trefoil fold ($H_{CC}$; PDB: accession number 3BTA) (Lacy *et al*., 1998). (**b**) Schematic representation of CNTs. The active holotoxins are formed by a 50 kDa L chain linked via a disulfide bond to a 100 kDa H chain. The carboxy-terminal portion $H_C$ (50 kDa) is responsible for neurospecific binding, whereas the amino terminus $H_N$ (50 kDa) is involved in the translocation of the catalytic domain into the cytosol. Structurally, $H_C$ can be subdivided in two 25 kDa domains, $H_{CN}$ and $H_{CC}$. $H_{CN}$ has structural analogies to the carbohydrate binding moiety of plant lectins and other oligosaccharide-binding proteins. $H_{CC}$ adopts a modified β-trefoil fold, which is present in several proteins involved in recognition and binding functions such as trypsin inhibitors. (**c**) Crystal structure of TeNT L chain (PDB accession number: 1YVG)(Rao *et al*., 2005) and $H_C$ chain. (PDB accession number: 1AF9) (Umland *et al*., 1997). Direct comparison between the L chains of TeNT, BoNT/A, /B, and /E suggest that their overall structure is very similar, differing only in terms of stringent substrate specificity. Similarly, the $H_{CN}$ domains of TeNT and BoNT/A display an overall identical fold, whereas the $H_{CC}$ of different CNT vary in their gangliosides interaction loops (arrowheads).

Å resolution, BoNT/A was found to be an elongated molecule, presenting a linear arrangement of three domains (Lacy *et al*., 1998; Stevens *et al*., 1991). These domains are structurally distinct with the exception of a large loop in the amino-terminal part of the H chain, which is wrapped around the perimeter of the L chain.

The $H_C$ domain, which is responsible for the neurospecific binding of CNT (Bizzini *et al*., 1977; Halpern and Neale, 1995; Herreros *et al*., 2000a), is composed of two distinct subdomains, rich in β-stucture and of roughly the same size. The amino-terminal subdomain ($H_{CN}$) has structural analogies with the carbohydrate binding moiety of plant lectins and other oligosaccharide-binding proteins. The carboxy-terminal portion ($H_{CC}$) of the H chain adopts a modified β-trefoil fold, which is present in several proteins involved in recognition and binding functions such as trypsin inhibitors. The entire $H_C$ domain remains completely isolated from the rest of the molecule, such that all the surface loops are accessible and therefore available for binding. Comparison of the crystal structures of different $H_C$ domains showed that the major differences between CNT resides in the loops of $H_{CC}$, where the sequence is poorly conserved (Umland *et al*., 1997). The removal of the amino-terminal portion $H_N$ of the H chain does not affect toxin binding, whereas the deletion of only a few residues from the carboxy-terminus abolishes toxin binding (Halpern and Loftus, 1993).

Studies with native and recombinant TeNT have shown that $H_C$ contains ganglioside- and cell-binding sites (Figueiredo *et al*., 1997). Two binding sites in $H_C$ have been found, which account for their interaction with gangliosides (Rummel *et al*., 2003). The identified interaction sites in gangliosides of the $G_{1b}$ series are absent in $G_{M1}$, which is consistent with its very low binding affinity to $H_C$.

The amino-terminal part of the H chain ($H_N$) is implicated in the pH-dependent membrane-penetration and translocation of the catalytic domain into the cytosol. It is composed of a loop interacting with the L

chain and a central body containing two very long α-helices as the main structural units. Although the function of this domain in the membrane insertion and L chain translocation has been well described (Donovan and Middlebrook, 1986; Shone *et al*., 1987), the exact mechanism(s) involved are still poorly understood. The amphipathic segment 659–681 of BoNT/A increased the permeability of lipid bilayers, implicating this fragment in pore formation (Oblatt-Montal *et al*., 1995). In the structure, the sequence corresponding to this peptide adopts a β-strand-like conformation and lies against the two main α-helices. This may suggest an involvement of this portion of the molecule in a pH-dependent conformational change, causing the exposure of previously hidden hydrophobic surfaces.

The L chain is the catalytic domain, responsible for the intracellular activity of the toxin (Ahnert-Hilger *et al*., 1989; Bittner *et al*., 1989; Mochida *et al*., 1989; Penner *et al*., 1986; Poulain *et al*., 1988; Weller *et al*., 1991). The L chain contains the catalytic zinc atom, essential for CNT endopeptidase activity. One zinc-atom is bound to the L chain of TeNT, BoNT/A, B and F (Schiavo *et al*., 1992a; Schiavo *et al*., 1992b; Schiavo *et al*., 1993a), while BoNT/C binds two atoms of zinc with different affinities (Schiavo *et al*., 1995). Heavy metal chelators are effective to remove the bound zinc and generate inactive apo-neurotoxins (Bhattacharyya and Sugiyama, 1989; Schiavo *et al*., 1992a).

The structure of BoNT/A, /B, and /E light chains suggest that substrate recognition cannot occur at the active sites of these CNT because their catalytic pocket organization and geometries are nearly identical (Agarwal *et al*., 2004; Lacy *et al*., 1998). The catalytic site is located deep in the structure of the protein, accessible only by a large channel, which accommodates the substrate. In the di-chain CNT, the entry to the channel is partially blocked by the large loop wrapping the L chain, and by the translocation domain itself (Lacy *et al*., 1998). Biochemical and mutagenesis studies have identified two critical residues in BoNT/A, $His_{222}$ and $His_{226}$, where the zinc atom is held in place. These residues are located in the sequence His-Glu-x-x-His, the characteristic zinc endopeptidase motif (Jongeneel *et al*., 1989; Schiavo *et al*., 1992a; Schiavo *et al*., 1992b; Wright *et al*., 1992). The glutamic acid residue in this motif coordinates the water molecule necessary for the catalysis (third ligand), whereas another glutamic acid ($Glu_{261}$ in BoNT/A) is the fourth ligand.

## Neurospecific binding

CNT binding has been examined using morphological and biochemical techniques in different systems like primary neuronal cultures, cell lines, and various membrane preparations (Halpern and Neale, 1995; Schiavo *et al*., 2000). CNT bind to the presynaptic membrane of cholinergic nerve terminals (Halpern and Neale, 1995). TeNT may also bind to sympathetic and adrenergic nerve fibres (Rossetto *et al*., 2001). *In vitro*, CNT are capable of binding to a variety of non-neuronal cells, but only at concentrations several orders of magnitude higher than those clinically relevant. In contrast, *in vivo* binding of CNT is absolutely neurospecific and requires concentrations in the subnanomolar range (Halpern and Neale, 1995; Simpson, 2000). The determinants of this high-affinity neurospecific binding are encoded within the $H_C$ domains, as recombinant $H_C$ fragments can counteract paralysis induced by the parental toxins (Lalli *et al*., 1999).

CNT bind to polysialogangliosides, in particular to members of the $G_{1b}$ series ($GD_{1b}$, $GT_{1b}$, and $GQ_{1b}$) (Halpern and Neale, 1995; Montecucco, 1986), with BoNTs containing one and TeNT two oligosaccharide-binding sites within the carboxy-terminal part of the $H_C$ domain (Rummel *et al*., 2003; Rummel *et al*., 2004b). The binding of CNT to polysialogangliosides is physiologically relevant, because mutations in the carbohydrate binding pockets decrease binding to the neuronal membrane (Louch *et al*., 2002; Rummel *et al*., 2003; Rummel *et al*., 2004b). Preincubation of NMJ preparations with $G_{1b}$ protects the NMJ from BoNT-dependent inhibition of neurotransmitter release and partially abolishes retrograde transport of TeNT (Stoeckel *et al*., 1977). Also, the removal of sialic acid residues from the membrane surface with neuraminidase decreases but does not abolish CNT activity (Bigalke *et al*., 1986). In addition, impairment of ganglioside biosynthesis leads to a reduced CNT activity *in vivo* (Kitamura *et al*., 1999; Williamson *et al*., 1999). Niemann (1991) suggested a model in which polysialogangliosides act as peripheral receptors for TeNT, mediating its retrograde transport to the CNS, where it binds to a second different acceptor (Niemann, 1991). Although appealing, this model suffers from the drawback of the low affinity and low specificity of polysialogangliosides as the only TeNT receptor in the periphery, where a high-affinity interaction is required to account for the extremely low doses of TeNT causing clinical symptoms *in vivo*. In addition, lack of binding competition between TeNT and BoNTs and their absolute neurospecificity make it unlikely that gangliosides are the unique binding determinant for CNT. Accordingly, specific protein co-receptors have been identified for some of the CNT. For example, BoNT/A, /B, /E, and /G interact with synaptotagmins I and/or II (Dong *et al*., 2003; Li and Singh, 1998; Rummel *et al*., 2004a; Yowler *et al*., 2002), which function as calcium-sensors at the synapse.

BoNT/B binds to the intraluminal portion of glycosylated synaptotagmin I and II (Nishiki *et al*., 1996) in a ganglioside-dependent manner. During neurotransmitter release, this region is exposed to the extracellular milieu, and it becomes accessible to large extracellular ligands. Thus, BoNTs might use synaptic vesicle (SV) endocytosis and recycling for their entry into MN. Moreover, the acidification of SV, which is necessary for their reloading with neurotransmitters, might mediate the acid-driven insertion of the heavy chain (Koriazova and Montal, 2003; Schiavo *et al*., 2000) into the SV membrane and the translocation of the L chain into the NMJ cytoplasm. However, the role of synaptotagmins as physiological BoNT receptors remains controversial because antibodies against this synaptotagmin domain fail to antagonize the binding and activity of BoNT/B at the NMJ (Bakry *et al*., 1997). Moreover, competition experiments clearly demonstrated that different BoNT serotypes do not share the same receptor (Evans *et al*., 1986; Habermann and Dreyer, 1986).

TeNT binds to one or more glycosylphosphatidylinositol (GPI)-anchored protein(s) of ~15 kDa (Herreros *et al*., 2000b). One of these proteins has been identified as Thy-1 in nerve growth factor (NGF) differentiated phaeochromocytoma (PC12) cells. However, it is unlikely to be the main protein receptor *in vivo* because Thy-1 knockout mice retain sensitivity to TeNT (Herreros *et al*., 2001). In contrast, pretreatment with a phosphatidylinositol-specific phospholipase, which cleaves the lipid anchor of GPI-anchored proteins, protects neurons from TeNT intoxication (Munro *et al*., 2001).

GPI-anchored proteins, cholesterol, gangliosides, and other sphingolipids are enriched in microdomains of the plasma membrane, termed *lipid rafts*. They act as platforms for signaling, ligand recognition, sorting, and endocytosis (Pike, 2003; Tsui-Pierchala *et al*., 2002). In addition, lipid rafts provide efficient and dynamic means for receptor clustering and ligand oligomerization. These include virulence factors, when locally concentrated on restricted domains of the plasma membrane (Abrami *et al*., 2003). Consequently, lipid microdomains are exploited by pathogens and virulence factors for their entry into host cells (Abrami *et al*., 2003; Duncan *et al*., 2002).

The complexity of the CNT receptors, which are composed of multiple lipid and protein components, together with the high affinity of CNT for the neuronal membrane has led to the recent proposal that arrays of presynaptic receptors (APRs) are involved in CNT binding (Montecucco *et al*., 2004). APRs are suggested to be dynamic entities that share several features with lipid rafts. Due to their high concentration at the NMJ and their lateral mobility, gangliosides are suggested to be the initial binding factors. They would act as "antennas" to capture CNT on the presynaptic membrane and allow subsequent clustering with the other molecules of the APR, thus leading to virtually irreversible binding. In addition to gangliosides, an APR would contain additional lipids, such as cholesterol, one or more GPI-anchored protein(s), and transmembrane protein(s). They may also include signaling molecules that upon binding trigger the internalization and/or sorting of CNT to specific synaptic structures. The APR recognized by BoNTs would guide them inside vesicles that acidify within the NMJ, whereas the APR binding TeNT would sort the neurotoxin into an endocytic vesicle undergoing retrograde transport along the axon. Upon sorting in the soma of the MN, TeNT will be released into the intersynaptic space, where it will enter inhibitory interneurons, possibly via SV endocytosis (Matteoli *et al*., 1996).

To reach its final site of action, TeNT has to bind to and enter two different neurons: a peripheral MN and an inhibitory interneuron of the spinal cord. In this regard, several proteins are taken up efficiently by MN when linked to recombinant TeNT $H_C$ (Coen *et al*., 1997; Fishman *et al*., 1990; Francis *et al*., 1995; Miana-Mena *et al*., 2002), as discussed in "Protein and gene transfer via TeNT." The identification of the receptors for TeNT present on MN will provide crucial information on this trafficking pathway leading from the NMJ to interneurons, offering new insights for the delivery of biologic and pharmacological agents into the spinal cord.

## INTERNALIZATION

Because the catalytic activity of the L chains is directed towards intracellular targets, this toxin domain must at least reach the cytosol. *In vivo*, CNT do not enter the cytosol directly via the plasma membrane but are endocytosed by the cell into vesicular compartments. Electron microscopic studies have shown that, after binding, CNT enter the lumen of vesicular structures in a temperature- and energy-dependent process (Critchley *et al*., 1985; Parton *et al*., 1987). In dissociated spinal cord neurons, gold-labeled TeNT is found first in coated pit structures at the cell surface, and then in a variety of vesicular and tubular structures as well as multivesicular bodies and, to a lesser extent, SV (Parton *et al*., 1987). In contrast, Montesano (1982) found that in liver cells TeNT accumulates in uncoated pits and non-clathrin coated vesicles. These discrepancies are difficult to reconcile and are probably a consequence of the different cell system and the high

concentration used by one group (Montesano *et al*., 1982).

It is well established that nerve stimulation facilitates intoxication by CNT (Habermann *et al*., 1980; Kryzhanovsky, 1958; Ponomarev, 1928; Schmitt *et al*., 1981; Wellhoner *et al*., 1973), and following membrane depolarization, TeNT colocalizes with SV markers in hippocampal neurons (Matteoli *et al*., 1996). In agreement with these findings, recent studies showed that TeNT uptake is dependent on presynaptic activity in MN *in vivo* (Miana-Mena *et al*., 2002), and that BoNT/A and /E enter neurons in a calcium- and stimulation-dependent manner (Keller *et al*., 2004). Generally, a high rate of neuroexocytosis correlates with a high rate of SV recycling via endocytosis, the two processes being tightly coupled (Murthy and De Camilli, 2003). Thus, a possible explanation for the shorter onset of paralysis induced by CNT under nerve stimulation is that they enter the synaptic terminal inside the lumen of SV. The accessibility of the internal lumen of SV to extracellular agents during neurotransmitter release has been demonstrated by the binding and uptake of antibodies specific for luminal epitopes of a SV protein (Kraszewski *et al*., 1995; Matteoli *et al*., 1992; Mundigl *et al*., 1995). According to this hypothesis, CNT might use SV as Trojan horses to gain entry to CNS neurons.

Several distinct mechanisms of endocytosis have been described (Conner *et al*., 2003), of which clathrin-mediated internalization is to date the best characterized. Additional pathways include caveolin-dependent endocytosis, as well as several clathrin- and caveolin-independent mechanisms. Though there may be additional ways of retrieval, the role of clathrin-mediated endocytosis in SV recycling is well established (Murthy and De Camilli, 2003). Therefore, it is likely that CNT exploit this pathway of clathrin-mediated endocytosis for their entry into the neuron in which they block release. This is an attractive model to explain the endocytosis of CNT in central neurons, but it is unlikely that SV recycling is responsible for the uptake of TeNT at the NMJ. In fact, three experimental findings contrast with this hypothesis. First, high frequency stimulation increases the rate of intoxication but not the binding of TeNT to the NMJ (Schmitt *et al*., 1981). If the toxin receptor is exposed during neuroexocytosis to allow the toxin to bind and then be endocytosed, an increase in the stimulation rate should also increase the total number of binding sites present at the NMJ. Second, TeNT is not active on a NMJ maintained at 18°C even in the presence of high-frequency stimulation and massive neurotransmitter release, whereas it is fully inhibitory at 25°C (Schmitt *et al*., 1981). Third, the uptake and retrograde transport of TeNT occur in NMJ intoxicated with BoNT/A, where neurotransmitter release is completely blocked (Habermann and Erdmann, 1978). The latter fact is in agreement with the notion that retrograde transport of various substances, including horseradish peroxidase, is not impaired in silenced NMJ (Kemplay and Cavanagh, 1983; Kristensson and Olsson, 1978). In addition, experiments performed in MN indicate that there is very limited co-localization between TeNT and SV markers (Lalli, 2002b), thus indicating that the pathway of internalization and intracellular trafficking of TeNT may be very different in peripheral and central neurons. Preliminary data suggest that in spinal cord MN, TeNT is also internalized via a clathrin-dependent pathway, which is, however, distinct from SV endocytosis (K.D., unpublished data).

## AXONAL TRANSPORT IN SPINAL CORD MOTOR NEURONS

Axons can reach a length of up to several meters. To overcome these distances, neurons have developed an exquisitely efficient and highly regulated transport system, which is essential for survival and growth, and for long-distance communication (Goldstein and Yang, 2000). Axonal transport was first observed in transected axons (Waller, 1852) and then confirmed in ligation experiments (Cajal, 1928; Weiss and Hiscoe, 1948). More recent metabolic radiolabeling studies clearly defined two types of transport systems, fast and slow. The fast component (0.1–5 μm/s) is mainly used for vesicle and organelle transport, and for secreted molecules like neurotrophins. The slow component (1–50 nm/s) is responsible for the transport of microtubules (MT), neurofilament components, cytoplasmic proteins, and metabolic enzymes (Vallee and Bloom, 1991).

Neurotrophins, their low affinity receptor ($p75^{NTR}$) (Lalli and Schiavo, 2002a), and several endocytic compartments use retrograde axonal transport from the synapse to the cell body to reach their final destination (Goldstein and Yang, 2000; Vallee and Bloom, 1991; Whitmarsh and Davis, 2001). Several pathogens, such as *Herpes* and *Polio* viruses, are targeted to the cell body, where the virus replicates or enters latency (Bearer *et al*., 2000; Satpute-Krishnan *et al*., 2003).

## TRANSPORT OF CLOSTRIDIAL NEUROTOXINS

In contrast to BoNTs, which remain at the synaptic site, TeNT carriers are recruited to the retrograde axonal

transport pathway towards the MN cell body located in the spinal cord (Stöckel *et al*., 1975). TeNT carriers are shared by both NGF and its low affinity neurotrophin receptor $p75^{NTR}$ (Lalli and Schiavo, 2002a). Despite the importance of these axonal compartments in health and disease, not much is known about their molecular and biophysical composition. Recently, we developed an assay based on fluorescently labeled TeNT $H_C$ to visualize and analyze the axonal retrograde transport in MN in real time (Figure 20.3a) (Lalli and Schiavo, 2002a).

Using this probe, three classes of TeNT carriers have been detected: round vesicles, tubules (Lalli and Schiavo, 2002a), and "vesicle clusters," which have a pearl-like morphology (S.B., unpublished results). Vesicles progress discontinuously with frequent pauses during their transport. In contrast, tubules and vesicle clusters display an overall faster and more continuous movement (Lalli and Schiavo, 2002a) (Figures 20.3b and 20.3c). Kinetic analysis revealed striking similarities between TeNT $H_C$ carriers and TeNT transport *in vivo* (0.8–3.6 μm/s) (Lalli *et al*., 2003b; Schiavo *et al*., 2000). These findings indicate that the non-toxic fragment TeNT $H_C$ can be used as a tool to dissect neuronal transport.

We have prepared chimeras of TeNT $H_C$ and ratiometric pHluorin, a pH sensitive green fluorescent protein mutant (Miesenbock *et al*., 1998), to determine the pH of retrogradely transported carriers in living MN. This issue is particularly interesting because acidic pH triggers a conformational change in TeNT and other CNT, which allows the active subunit to translocate through the carrier membrane into the cytosol (Montecucco *et al*., 1994; Williamson and Neale, 1994). During transport, TeNT $H_C$ carriers display neutral pH, whereas stationary TeNT $H_C$ organelles exhibit a wide spectrum of pHs, ranging from acidic to neutral. This distinct pH regulation is dependent upon a differential targeting of the vacuolar ($H^+$) ATPase, which is likely to be excluded from the majority of TeNT $H_C$-positive compartments (Bohnert and Schiavo, 2005). These findings suggest that TeNT $H_C$ exploits an endocytic transport pathway, which escapes acidification and targeting to degradative organelles.

Crucial components of the axonal transport machinery are motor proteins that use ATP to perform mechanical work. The main component of neuronal traffic is MT-based (Goldstein and Yang, 2000). However, actin dependent motors are also relevant for neuronal transport, suggesting a functional connection between

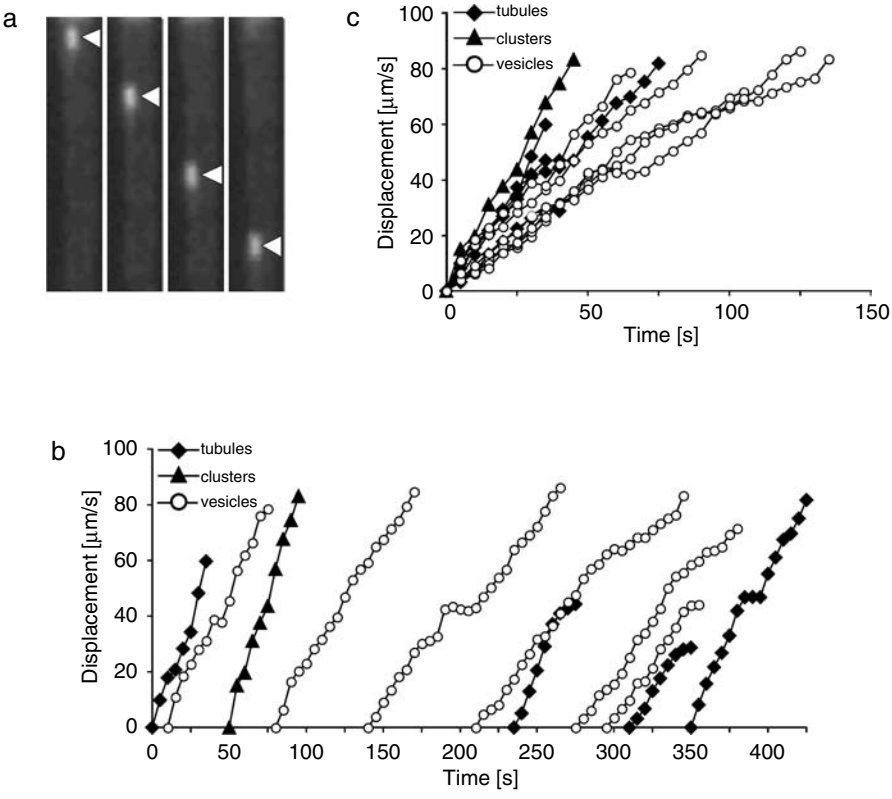



**FIGURE 20.3** Kinetics of axonal retrograde transport of TeNT $H_C$ carriers (**a**) TeNT $H_C$ containing organelles are retrogradely transported along a MN axon. Time series of a carrier containing fluorescently-labeled TeNT $H_C$ is shown. The cell soma is out of view located to the bottom. Frames have been taken every 5 s. (**b**) Displacement graph of TeNT $H_C$ carriers. The movements of one vesicle cluster (triangles), four tubules (diamonds), and six vesicles (empty circles) imaged during a representative experiment have been tracked and plotted versus time. Vesicular carriers display an overall slower and more discontinuous movement than the faster vesicle cluster and tubules. (**c**) The starting time for all carriers has been set as time = 0. The intervals between time points are 5 s.

MT and actin-based transport (Bridgman, 1999; Kamal and Goldstein, 2000).

Three families of force generating ATPases, myosins, kinesins, and dynein have been identified in neurons (Vale, 2003). Myosin I and II move along actin filaments from the minus to the plus end. Kinesins and dynein are ATPases, which are stimulated by binding to MT. Most kinesins move towards the MT plus end, whereas dynein moves in the opposite direction. Therefore, kinesins are thought to be responsible for anterograde transport (Griffin and Watson, 1988), and dynein for retrograde MT-based transport in axons (Schnapp and Reese, 1989; Vallee *et al*., 1989).

Dynein plays a main role in TeNT transport. For example, in *Loa* mice, a mutation in the motor protein dynein is associated with axonal transport defects and MN degeneration (Hafezparast *et al*., 2003). Cytoplasmic dynein binds directly to a fragment of neurofilament subunit M. Therefore, cytoplasmic dynein is thought to play a role in the short-duration retrograde movement of neurofilaments undergoing slow anterograde transport along axonal MT (Wagner *et al*., 2004). Dynein may also regulate other molecular motors because its inhibition affects the contribution of kinesins and myosins to retrograde transport (Lalli *et al*., 2003a).

Kinesins are a large superfamily of MT-dependent motor proteins. Three subfamilies have been identified: the Kin C family comprises kinesins with carboxy-terminal located core motor domain; Kin N kinesins have an amino-terminal core motor domain; and Kin I kinesins have an internal core motor domain. Kin N kinesins have been named "conventional kinesins" and Kin C and Kin I "unconventional kinesins." Kin N kinesins bind to MT and move from the minus-MT end towards the plus-MT end, whereas Kin C kinesins walk in the opposite direction. In nerve cells, conventional kinesins are responsible for the transport of organelles from the cell body to the nerve endings in both axons and dendrites. Interestingly, the presence of soluble kinesin and accessory factors is not required for anterograde movement (Schnapp *et al*., 1992). However, pharmacological inhibition of kinesin slows both anterograde and retrograde axonal transport (Brady *et al*., 1990; Lalli *et al*., 2003a). Moreover, mutations in kinesin heavy chain cause some forms of motor neuron disease, including amyotrophic lateral sclerosis, by disrupting fast axonal transport in both directions (Hurd and Saxton, 1996). The retrograde movement of TeNT $H_C$ carriers in MN treated with a potent kinesin inhibitor was strongly reduced (Lalli *et al*., 2003a).

Why does the disruption of a unidirectional motor system lead to bidirectional defects? The reason for this unexpected finding lies in the interdependence of anterograde and retrograde axonal transport systems, which is confirmed by the fact that dynein and kinesin share communal accessory factors, such as the dynactin complex (Martin *et al*., 1999). Therefore, dynein might not only be the main motor of fast retrograde axonal transport, but also a regulator of other motors associated with TeNT $H_C$ transport (Lalli *et al*., 2003a).

In addition to MT-dependent motors, the retrograde axonal transport of TeNT also requires axonal myosins, which move on F-actin (Cao *et al*., 2004). In particular, myosin Va has been shown to be important for retrograde transport of TeNT. MN from myosin Va-null embryos displayed a slower TeNT retrograde transport than wild-type cells (Lalli *et al*., 2003a).

## TRANSLOCATION OF CNT THROUGH VESICLE MEMBRANES

The different trafficking and targeting of TeNT and BoNTs indicate that neurotoxin internalization and translocation of the L chains are not directly linked. In order to display their catalytic activity, the L chains of CNT have to cross the vesicle membrane to reach the cytosol. CNT have to be exposed to a low pH to induce nerve intoxication (Matteoli *et al*., 1996; Simpson, 1982; Simpson *et al*., 1994; Williamson and Neale, 1994), even though the application of L chains to the cytosol is sufficient to block neurotransmitter release (Ahnert-Hilger *et al*., 1989; Bittner *et al*., 1989; Mochida *et al*., 1989; Penner *et al*., 1986; Poulain *et al*., 1988; Weller *et al*., 1991). It is therefore likely that an acidic pH is crucial to enable the active subunit to translocate through the vesicle's membrane into the cytosol (Montecucco *et al*., 1994).

Studies on model membranes (Montecucco *et al*., 1994) and in cell cultures (Beise *et al*., 1994) have shown that low pH induces a conformational change in CNT from a hydrophilic neutral to a hydrophobic acidic form. This transition enables both the H and L chains to penetrate into the hydrophobic core of the lipid bilayer (Boquet and Duflot, 1982; Cabiaux *et al*., 1985; Menestrina *et al*., 1989; Montecucco, 1986; Montecucco *et al*., 1989; Schiavo *et al*., 1990b). At low pH, CNT form a cation-selective ion channel in planar lipid bilayers with conductance of a few tens of picosiemens that are permeable to molecules smaller than 700 Da. CNT channels may be formed by oligomerization of the $H_N$ domain (Donovan and Middlebrook, 1986; Menestrina *et al*., 1989; Schmid *et al*., 1993; Shone *et al*., 1987), which is built by a pair of 105 Å-long α-helices buried at neutral pH. These helices are flanked by a short amphi-

pathic segment, which is able to form channels with similar properties to the holotoxin (Montal *et al*., 1992; Oblatt-Montal *et al*., 1995). It was proposed that the channel is formed by a toxin tetramer bringing four of these amphipathic segments into strict proximity with their hydrophobic residues lining the lumen of the pore (Montal *et al*., 1992; Oblatt-Montal *et al*., 1995). This is in agreement with the three-dimensional image reconstruction of the channel formed by BoNT/B in phospholipids bilayers (Schmid *et al*., 1993).

Both H and L chains are supposed to change conformation at low pH. The acidic toxin form may have the properties of a molten globule (Bychkova *et al*., 1988; van der Goot *et al*., 1991). The heavy chain forms a hydrophilic transmembrane cleft, nesting the passage of the partially unfolded L chain with its hydrophobic segments facing the lipids. The cytosolic neutral pH induces the L chain to refold and regain its water-soluble neutral confirmation following reduction of the interchain disulfide bond. Cytosolic chaperones may be involved in facilitating the exit of the L chain from the vesicle membrane and in promoting its cytosolic refolding (Ratts *et al*., 2003). The latter process is complicated by the absolute requirement of zinc for L chain catalytic activity. The protonation of the histidines coordinating the zinc ion at low pH is expected to release the metal atom, which has to be acquired again in the cytosol.

As the L chain is released into the cytosol, the transmembrane hydrophilic cleft of the H chain tightens up to reduce the amount of hydrophilic protein surface exposed to the hydrophobic core of the membrane. However, this process is not complete, leaving a channel across the membrane that is proposed to be the structure responsible for the ion conducting properties of CNT. Pore formation is therefore a consequence of membrane translocation, rather than its prerequisite (Schwab *et al*., 2000).

## INTRACELLULAR ZINC-ENDOPEPTIDASE ACTIVITY

The catalytic nature of CNT was discovered following the observation that the L chains contain the His-Glu-x-x-His binding motif of zinc-endopeptidases (Kurazono *et al*., 1992; Schiavo *et al*., 1992a; Schiavo *et al*., 1992b; Wright *et al*., 1992). Following this observation, it was soon demonstrated that TeNT was blocking ACh release at *Aplysia californica* synapses via a zinc-dependent protease activity (Schiavo *et al*., 1992a).

CNT are remarkably specific proteases. Only three targets, all members of the SNARE superfamily (Bock and Scheller, 1997), have been identified. TeNT and BoNT/B, /D, /F, and /G cleave VAMP (or synaptobrevin) but each at different sites (Figure 20.4) (Schiavo *et al*., 1992c; Schiavo *et al*., 1994; Schiavo *et al*., 1993b; Schiavo *et al*., 1993a; Yamasaki *et al*., 1994b; Yamasaki *et al*., 1994c); BoNT/A and /E cleave SNAP-25 at two different sites; and BoNT/C cleaves both syntaxin and SNAP-25 (Binz *et al*., 1994; Blasi *et al*., 1993a; Blasi *et al*., 1993b; Foran *et al*., 1996; Osen-Sand *et al*., 1996; Schiavo *et al*., 1993b; Schiavo *et al*., 1993c; Schiavo *et al*., 1995; Vaidyanathan *et al*., 1999; Williamson *et al*., 1996) (Figure 20.4).

SNARE proteins have been implicated as crucial components in most, if not all, intracellular membrane trafficking events (Chen and Scheller, 2001; Jahn and Sudhof, 1999). In eukaryotic cells, molecules need to be delivered to their correct intracellular destinations maintain the structural integrity of cellular compartments. To achieve this, transport vesicles bud from a donor organelle and then dock and fuse with a target membrane. SNARE were originally divided into v-SNARE and t-SNARE, based on their vesicle or target membrane location (Sollner *et al*., 1993). SNARE are currently classified into Q- and R-SNARE, depending on the presence of either glutamine or arginine in a

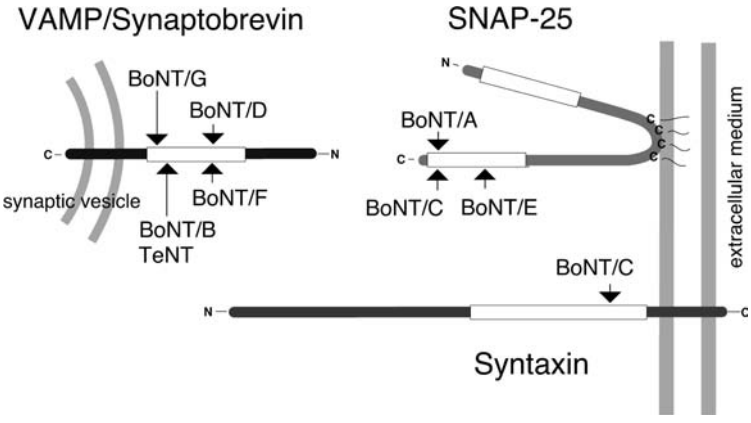



**FIGURE 20.4** Schematic structure of SNARE proteins cleaved by CNTs. VAMP is a type-II membrane protein anchored to SSV by a single transmembrane domain. VAMP is characterized by a short intraluminal domain, a conserved 60 residue-long cytosolic portion, which is able to form coiled coils, and a poorly conserved amino terminus. SNAP-25 is bound to the presynaptic membrane by several palmitoylated cysteines located in the middle of the molecule. Syntaxin is anchored by a single transmembrane domain to the neuronal presynaptic plasma membrane. The amino terminal part is cytosolic and only a few residues protrude into the intersynaptic cleft. The arrows indicate the sites of CNT cleavage.

central position of the SNARE motif (Jahn and Sudhof, 1999).

Studies on yeast and mammalian SNARE, anchored to liposomes, have reinforced the concept that the specificity of membrane fusion events relies on a precise pattern of v-SNARE–t-SNARE interactions (Bonifacino and Glick, 2004). Unique receptors of SNARE are found in different specific cellular compartments, suggesting that SNARE are functionally involved in all vesicle trafficking steps in endocytotic cells. Although the specificity of intracellular membrane fusion is associated with the biophysical properties of SNARE, a better spatial and temporal control of SNARE-mediated fusion is provided by additional regulatory systems, such as Rab GTPases and other proteins helping vesicle tethering and/or SNARE activation.

## CLOSTRIDIUM NEUROTOXIN TARGETS

### SNAP-25

SNAP-25 (synaptosomal-associated membrane protein of 25 kDa) was originally described as the major palmitoylated protein in the CNS (Hess *et al*., 1992; Oyler *et al*., 1989; Wilson *et al*., 1996). As shown in Figure 20.4, this protein lacks a classical transmembrane segment and its membrane binding is thought to be mediated by the palmitoylation of several cysteines in the middle of the polypeptide chain (Hess *et al*., 1992; Veit *et al*., 1996). SNAP-25 interacts with the other t-SNARE, syntaxin, and with VAMP to form the synaptic SNARE complex, which constitutes the core of the neuroexocytic apparatus (Sollner *et al*., 1993). The SNAP-25 family contributes two of the four α-helices that compose the SNARE complex. Upon interaction with syntaxin, SNAP-25 forms a three-helix bundle complex, which may act as a VAMP/synaptobrevin receptor on the plasma membrane (An and Almers, 2004). In addition, SNAP-25 forms a stochiometric complex with the SV calcium sensor synaptotagmin (Bai and Chapman, 2004), and this interaction is believed to be important in a late step of the calcium-dependent phase of neurotransmitter release (Banerjee *et al*., 1996; Schiavo *et al*., 1997).

SNAP-25 is required for axonal growth during neuronal development and in nerve terminal plasticity in the mature nervous system (Geddes *et al*., 1990; Osen-Sand *et al*., 1993) and is conserved from yeast to humans (Wilson *et al*., 1996). It is expressed in the nervous system in two developmentally regulated isoforms (SNAP-25A and B), with the B form predominating in the adult nervous tissue. Both a shorter (SNAP23) and a longer (SNAP29) isoform are also known that can partially vicariate for SNAP-25 function (Sorensen *et al*., 2003). In mast cells, SNAP-23 is required for exocytosis, implying a crucial role of this isoform in promoting membrane fusion (Guo *et al*., 1998).

### Syntaxin

Syntaxins are widely distributed throughout the cell and their function is required for a wide range of intracellular membrane fusion pathways (Teng *et al*., 2001). They belong to a family of proteins that are tail-anchored (also called type IV membrane proteins) (Salaun *et al*., 2004). Syntaxins have an amino-terminal cytoplasmic domain that is membrane bound by virtue of a single carboxy-terminal hydrophobic domain with no ectodomain (Borgese *et al*., 2003) (Figure 20.4). Syntaxins constitute a large protein family with homologues in yeast and plants. There are 15 members of the syntaxin family in the human genome, of which four are expressed in the plasma membrane. Syntaxin 1 is mainly located on the neuronal plasmalemma (Bennett *et al*., 1992; Inoue and Akagawa, 1992) and functions in regulated secretion and exocytosis. Syntaxin 2 and 3 are also functioning in exocytosis, whereas syntaxin 4 is involved in glucose transporter traffic in adipocytes.

Syntaxin 1 is associated with calcium channels in the active zones (Atlas, 2001; Stanley, 1997), but it is also present on most of the neuronal cell membrane (Garcia *et al*., 1995). It interacts in a calcium-dependent manner with some isoforms of the SV protein synaptotagmin (Sudhof, 1995) via a domain that is also responsible for the interaction with VAMP and α-SNAP (soluble NSF attachment protein) (Hayashi *et al*., 1995; Kee *et al*., 1995; Lin and Scheller, 1997). Together with SNAP-25, syntaxin 1 undergoes a recycling process in organelles indistinguishable from SV (Walch-Solimena *et al*., 1995). Syntaxins are essential for neuronal development and survival, because BoNT/C, unlike the other CNT, acts as a cytotoxic factor in neurons (Osen-Sand *et al*., 1996). Disruption of synaptic architecture by BoNT/C in central nervous system neurons activates distinct and independent neurodegenerative programs in the axo-dendritic network and in the cell bodies (Berliocchi *et al*., 2005). Several isoforms undergo a complex pattern of alternative splicing and expression during long-term potentiation, thus suggesting that syntaxins are involved in synaptic plasticity (Rodger *et al*., 1998). This differential expression could be important for a direct modulation of calcium entry via selective interaction with specific calcium channels, in addition to the formation of distinct SNARE complexes with different SNAP-25 and VAMP isoforms.

## VAMP

VAMP (vesicle-associated membrane protein or synaptobrevin) is a protein of 13 kDa localized to SV, dense core granules, and synaptic-like microvesicles, and is the prototype of vesicular SNARE (v-SNARE) (Sudhof, 1995). Several isoforms have been identified, which are present in all vertebrate tissues, their relative amount and distribution differing between tissues and cell-types (Rossetto *et al*., 1996; Rossi *et al*., 2004). Structurally, VAMP can be divided in to four functional domains (Baumert *et al*., 1989). The amino-terminal portion is proline rich and isoform specific, whereas the central portion of the cytoplasmic domain is very conserved through evolution and contains the coiled-coil region responsible for SNARE complex formation (Lin and Scheller, 1997; Sutton *et al*., 1998). The protein is anchored to the membrane by a single transmembrane domain, which is followed by a short and poorly conserved intravesicular portion (Figure 20.4). VAMP-2, the major SNARE protein of SV, is essential for two synapse-specific membrane trafficking steps, fast exocytosis for neurotransmitter release, and fast endocytosis that mediates rapid reuse of synaptic vesicles (Deak *et al*., 2004). Surprisingly, VAMP is not necessary for asynchronous neurotransmitter release, which is still present upon its cleavage by TeNT and BoNTs and in neurons derived from knockout mice (Humeau *et al*., 2000; Schoch *et al*., 2001).

Several experimental data support a direct correlation between CNT-induced proteolysis of SNARE and the inhibition of neurotransmitter release. Recombinant VAMP, SNAP-25, and syntaxin are cleaved at the same peptide bonds as the corresponding cellular proteins, indicating that no additional endogenous factors are necessary for the proteolytic activity of the CNT. Of the several isoforms of SNARE proteins identified in different species and tissues, only some of them are susceptible to proteolysis by CNT. In general, a SNARE protein may be resistant to a neurotoxin because of mutations at the cleavage site or in other regions involved in neurotoxin binding.

## BASIS FOR CLOSTRIDIUM NEUROTOXIN RECOGNITION OF SNARE PROTEINS

An inspection of the sequence of the three synaptic SNARE at the CNT cleavage sites reveals no conserved patterns that could account for the specificity of these zinc-proteases for their intraneuronal targets. Hence, whereas the overall architecture of the active sites of theses enzymes is expected to be similar, each CNT must differ in its detailed spatial organization in order to accommodate and hydrolyze such different peptide bonds.

Biochemical studies have uncovered several unique features of CNT. Short peptides encompassing the cleavage site are not cleaved, although they bind the toxin, while longer segments are cleaved (Foran *et al*., 1994; Yamasaki *et al*., 1994a). Strikingly, TeNT and BoNT/B cleave VAMP at the same peptide bond (Gln76-Phe77) (Schiavo *et al*., 1992c), yet when injected into the animal they cause the opposite symptoms of tetanus and botulism. This observation has been particularly relevant, because it clearly demonstrates that the distinct symptoms derive from different sites of intoxication rather than from a different intracellular mechanism of action. Even though TeNT and BoNT/B hydrolyze the same peptide bond of VAMP, they do have different requirements in terms of the maximal length of the peptide acting as a substrate. For example, the minimal VAMP segment cleaved by BoNT/B spans amino acids 44–94 but is extended to 33–94 for TeNT (Foran *et al*., 1994).

Some CNT hydrolyze a peptide bond, leaving identical sequences elsewhere in the substrate untouched. Other CNT have some degree of flexibility in terms of the peptide bond cleaved. BoNT/B cleaves Gln-Phe, present in the natural substrate VAMP, but is also able to hydrolyze peptides in which the above bond was substituted by Asn-Phe, Ala-Phe, or Gln-Tyr (Breidenbach and Brunger, 2004). BoNT/A and /C are poorly effective on isolated SNAP-25 and syntaxin, requiring the membrane insertion or their substrates for full activity (Blasi *et al*., 1993b; Schiavo *et al*., 1995). These findings indicate that the toxin-substrate interaction requires some other structural elements of the SNARE sequence serving as recognition motifs for the CNT.

Comparison of the sequence of synaptic SNARE proteins of different species shows the presence of a 10 residue-long motif, termed SNARE motif (Rossetto *et al*., 1994). This motif forms an α-helix with a negatively charged face flanked by a hydrophobic face (Rossetto *et al*., 1994; Washbourne *et al*., 1997). Multiple copies of this motif are present in syntaxin, SNAP-25, and VAMP and are proposed to act as CNT recognition sites. In fact, only peptides that include at least one SNARE motif are cleaved *in vitro* by CNT (Cornille *et al*., 1997; Foran *et al*., 1994). The SNARE motif is exposed on the protein surface on native non-assembled SNARE proteins, as shown by the binding of anti-SNARE motif antibodies. These antibodies cross-react among the three SNARE and inhibit the proteolytic activity of the neurotoxins (Pellizzari *et al*., 1996). CNT also cross-inhibit each other (Pellizzari *et al*., 1996), possibly by

interacting with the SNARE motif. Extensive site-directed mutagenesis of VAMP and SNAP-25 demonstrates that different CNT bind different SNARE motifs (Pellizzari *et al*., 1997; Pellizzari *et al*., 1996; Washbourne *et al*., 1997). Resistance to CNT *in vivo* is sometimes associated with mutations in the SNARE motifs or with deviations from the consensus. In fact, the drosophila VAMP lacks one of the three acidic residues in each SNARE motif, and is not cleaved by TeNT (Sweeney *et al*., 1995).

The molecular basis of the substrate recognition by CNT is however still under debate. The validity of a reported co-crystal structure of VAMP-2 bound to BoNT/B (Hanson and Stevens, 2000) has been questioned recently (Breidenbach and Brunger, 2004; Rupp and Segelke, 2001), mainly due to the lack of observed electron density for the substrate and the improbable ligand stereochemistry. Recently, Breidenbach and Brunger reported a novel high-resolution structure of a CNT L chain in complex with its SNARE substrate (Breidenbach and Brunger, 2004). To overcome the inability to co-crystallize wild-type BoNT/A L chain with SNAP-25, an inactive variant (E224Q, Y366F) has been used together with a truncated version (residues 141–204) of SNAP-25. This SNAP-25 fragment adopted three types of secondary structure in complex with BoNT/A: residues 147–167 formed a distorted α-helix, residues 168–200 were extended, whereas residues 201–204 were involved in a distorted β-sheet (Breidenbach and Brunger, 2004).

The amino-terminal helical region of the SNAP-25 fragment interacts with the L chain of BoNT/A along the hydrophobic patch formed at the interface of four α-helices, named the α-exosite. The carboxy-terminal portion of the SNAP-25 portion forms one strand of a distorted, three-stranded antiparallel β-sheet at a region that is referred to as β-exosite. For BoNT/A substrate recognition, SNAP-25 has to be bound to a presynaptic membrane via its linker domain. In the membrane bound, non-complexed SNAP-25, the amino- and carboxy-terminal domains are unstructured or flexible. The binding of BoNT/A is probably initiated by helix formation at the α-exosite. Two anchor points along the extended portion of SNAP-25, residues 170–172 and 192–193, and individual side chains in the nearby region are additional determinants of substrate specificity. These sites reduce $K_m$ and enhance binding of the β-exosite, inducing conformational changes at the active site, which make the endopeptidase competent to cleave its substrate.

BoNT/A can cleave small peptides (residues 192–206 of SNAP-25), which only bind to one anchor point in addition to the β-exosite, but at reduced $k_{cat}/K_m$ (Sukonpan *et al*., 2004). The lack of anchor points might therefore explain the different minimal residue segments of VAMP cleaved by BoNT/B (amino acids 44–94) and TeNT (amino acids 33–94) (Foran *et al*., 1994). Therefore, most of the enzyme-substrate interface serves to provide a substrate-specific boost to $k_{cat}/K_m$ by reducing $K_m$ (Breidenbach and Brunger, 2004). These findings also explain the inability of BoNT/A to cleave SNAP-25 when it is present in complex with other SNARE.

In conclusion, these studies suggest that multiple recognition sites on the SNARE proteins determine the specificity of the CNT for their targets. This binding is followed by interactions with other regions of the sequence that are different for each SNARE, including the segment containing the peptide bond to be cleaved. The relative contribution of these interactions for the specificity and strength of CNT binding to each SNARE remains to be determined.

## PROTEIN AND GENE TRANSFER VIA TENT

To date, the pharmacological treatment of brain diseases has been challenging because conventional drugs are mostly uneffective in crossing the blood-brain barrier (BBB). However, TeNT is able to enter the central nervous system by bypassing the BBB. This property makes TeNT a potentially exploitable resource for cargo delivery to the brain. The non-toxic carboxy-terminal $H_C$ fragment of TeNT is easy to handle, and it is the ideal transporter for proteins and DNA from the bloodstream to the CNS. However, a major drawback for the widespread use of TeNT in human therapy would be the immunity against tetanus due to standard vaccinations.

CuZn superoxide dismutase (SOD-1), an enzyme involved in the scavenging of superoxide free radicals, has been studied as a neuro-protectant to ameliorate oxidative injury. Increased levels of SOD-1 are cyto-protective in experimental models of neurological disorders associated with free radical toxicity, as found in strokes and traumas. A fusion-protein of SOD-1 and the non-toxic TeNT $H_C$ was internalized and retrogradely transported into cultured hippocampal neurons, and the hybrid protein remained enzymatically active after transport (Figueiredo *et al*., 1997; Francis *et al*., 2004b; Francis *et al*., 1995). Another hybrid protein, produced from a β-galactosidase gene (lacZ)-TeNT $H_C$ gene fusion can be retrogradely transported across a synapse *in vivo* (Coen *et al*., 1997). TeNT $H_C$ fused to the β-galactosidase enzyme reached second and higher-order neurons after injection in rat tongue (Miana-Mena *et al*., 2003). Furthermore, a fusion

construct of diphtheria toxin and TeNT $H_C$ has also been used to deliver passenger proteins to the neuronal cytosol (Francis *et al*., 2004a).

A somatic gene transfer approach based on TeNT $H_C$ was used to map muscle to MN projections in *Xenopus* spinal cord. DNA encoding the LacZ gene fused to TeNT $H_C$ could be produced from plasmid DNA injected into muscle, and the fusion protein was able to cross the NMJ and undergo retrograde transport *in vivo* (Coen *et al*., 1999). These results provided the first demonstration of the synthesis and transport of a TeNT $H_C$ fusion protein produced directly from exogenous DNA in a vertebrate system. Retrograde trans-synaptic transfer of a fusion protein of GFP and TeNT $H_C$, expressed in defined neurons in transgenic mice, was used to assess the genetic mapping of neuronal circuits (Maskos *et al*., 2002). Furthermore, TeNT $H_C$ has been shown to be suitable as targeting moiety for viral vectors, which indicates a potential for gene therapy of inherited neurodegenerative diseases, such as spinal muscular atrophy (Schneider *et al*., 2000). Taken together, TeNT $H_C$ chimeras could serve as effective tools to deliver important pharmacological cargoes to the CNS, providing an alternative therapeutical approach to neurological disorders.

## CLOSTRIDIUM NEUROTOXINS AND SIGNALING

Not all the clinical symptoms of tetanus intoxication can be attributed to the block of neurotransmission. Several symptoms such as insomnia, hyperactivity, and hyperthermia suggest the implication of different mechanisms, different neurons, and different areas of the CNS.

The binding of TeNT triggers the activation of specific signaling cascades (Gil *et al*., 2001), even before classical tetanus symptoms are evident (Aguilera *et al*., 1993; Gil *et al*., 1998). The $H_C$ fragment of TeNT activates different protein kinase C (PKC) isoforms as well as the Extracellular signal-Regulated protein Kinase/Mitogen-Activated Protein Kinase (ERK/MAPK) and the neurotrophin receptor TrkA (Gil *et al*., 2001). Furthermore, $H_C$ might enhance phospholipase C (PLC)γ-1 activity, an enzyme involved in calcium homeostasis, thus modulating intracellular calcium influx. In cultured cortical neurons, $H_C$ activates a signaling cascade involving Trk receptor, the survival pathway Akt, and the ERK1/2 pathway (Gil *et al*., 2003). TeNT has been shown to rescue cerebellar granule neurons from apoptosis by activating the PI3 Kinase/Akt pathway (Gil *et al*., 2003).

TeNT also specifically blocks $Na^+$-dependent serotonin (5-HT) uptake in rat-brain synaptosomes (Inserte *et al*., 1999; Najib *et al*., 1999). Serotonin transporter (SERT) is the sole molecule responsible for extracellular 5-HT transport (Borowsky and Hoffman, 1995). PKC has been implicated as a regulator of SERT activity via the phosphorylation of Ser/Thr residues responsible for re-uptake inhibition (Qian *et al*., 1997). This inhibition is independent of the metalloprotease activity of the L chain, and its potency is higher than 5-HT selective re-uptake inhibitors (Inserte *et al*., 1999; Najib *et al*., 1999).

TeNT-dependent signaling and its physiological consequences are still poorly understood. The fact that $H_C$ colocalizes with NGF and its low affinity receptor $p75^{NTR}$ in retrogradely transported carriers (Lalli and Schiavo, 2002a) raises the question of the physiological relevance of the signaling triggered by TeNT. Indeed, the neurotrophin signaling activated by TeNT is not restricted to the synapse but can also be propagated by retrograde transport within signaling endosomes (Kahn *et al*., 2002), which might contribute to the regulation of axonal transport (Chan *et al*., 2004; Mandelkow *et al*., 2004). Signaling molecules activated by TeNT might therefore act on different levels: internalization, transport, and/or sorting of the toxin.

## ACKNOWLEDGMENTS

We apologize to our colleagues whose work has been omitted due to space limitations. Work in the laboratories of the authors is supported by the European Molecular Biology Organisation (S.S.) and by Cancer Research UK.

## REFERENCES

Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003). Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. *J. Cell. Biol*. **160,** 321–328.

Agarwal, R., Eswaramoorthy, S., Kumaran, D., Binz, T. and Swaminathan, S. (2004). Structural analysis of botulinum neurotoxin type E catalytic domain and its mutant Glu212—>Gln reveals the pivotal role of the Glu212 carboxylate in the catalytic pathway. *Biochemistry*, **43,** 6637–6644.

Aguilera, J., Padros-Giralt, C., Habig, W.H. and Yavin, E. (1993). GT1b ganglioside prevents tetanus toxin-induced protein kinase C activation and down-regulation in the neonatal brain *in vivo*. *J. Neurochem*., **60,** 709–713.

Ahnert-Hilger, G., Weller, U., Dauzenroth, M.E., Habermann, E. and Gratzl, M. (1989). The tetanus toxin light chain inhibits exocytosis. *FEBS. Lett*., **242,** 245–248.

An, S.J. and Almers, W. (2004). Tracking SNARE complex formation in live endocrine cells. *Science*, **306,** 1042–1046.

Atlas, D. (2001). Functional and physical coupling of voltage-sensitive calcium channels with exocytotic proteins: ramifications for the secretion mechanism. *J. Neurochem.*, **77,** 972–985.

Bai, J. and Chapman, E.R. (2004). The C2 domains of synaptotagmin—partners in exocytosis. *Trends. Biochem. Sci.*, **29,** 143–151.

Bakry, N.M., Kamata, Y. and Simpson, L.L. (1997). Expression of botulinum toxin binding sites in Xenopus oocytes. *Infect. Immun.*, **65,** 2225–2232.

Banerjee, A., Kowalchyk, J.A., DasGupta, B.R. and Martin, T.F. (1996). SNAP-25 is required for a late postdocking step in Ca2+-dependent exocytosis. *J. Biol. Chem.*, **271,** 20227–20230.

Baumert, M., Maycox, P.R., Navone, F., De Camilli, P. and Jahn, R. (1989). Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *Embo. J.*, **8,** 379–384.

Bearer, E.L., Breakefield, X.O., Schuback, D., Reese, T.S. and LaVail, J.H. (2000). Retrograde axonal transport of herpes simplex virus: evidence for a single mechanism and a role for tegument. *Proc. Natl. Acad. Sci.* USA, **97,** 8146–8150.

Beise, J., Hahnen, J., Andersen-Beckh, B. and Dreyer, F. (1994). Pore formation by tetanus toxin, its chain, and fragments in neuronal membranes and evaluation of the underlying motifs in the structure of the toxin molecule. *Naunyn. Schmiedebergs Arch. Pharmacol.*, **349,** 66–73.

Bennett, M.K., Calakos, N. and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science*, **257,** 255–259.

Berliocchi, L., Fava, E., Leist, M., Horvat, V., Dinsdale, D., Read, D. and Nicotera, P. (2005). Botulinum neurotoxin C initiates two different programs for neurite degeneration and neuronal apoptosis. *J. Cell. Biol.*, **168,** 607–618.

Bhattacharyya, S.D. and Sugiyama, H. (1989). Inactivation of botulinum and tetanus toxins by chelators. *Infect. Immun.*, **57,** 3053–3057.

Bigalke, H., Muller, H. and Dreyer, F. (1986). Botulinum A neurotoxin unlike tetanus toxin acts via a neuraminidase sensitive structure. *Toxicon.*, **24,** 1065–1074.

Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T.C., Jahn, R. and Niemann, H. (1994). Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. *J. Biol. Chem.*, **269,** 1617–1620.

Bittner, M.A., Habig, W.H. and Holz, R.W. (1989). Isolated light chain of tetanus toxin inhibits exocytosis: studies in digitonin-permeabilized cells. *J. Neurochem.*, **53,** 966–968.

Bizzini, B., Stoeckel, K. and Schwab, M. (1977). An antigenic polypeptide fragment isolated from tetanus toxin: chemical characterization, binding to gangliosides, and retrograde axonal transport in various neuron systems. *J. Neurochem.*, **28,** 529–542.

Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T.C., Niemann, H. and Jahn, R. (1993a). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. *Nature*, **365,** 160–163.

Blasi, J., Chapman, E.R., Yamasaki, S., Binz, T., Niemann, H. and Jahn, R. (1993b). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *Embo J.*, **12,** 4821–4828.

Bock, J.B. and Scheller, R.H. (1997). Protein transport. A fusion of new ideas. *Nature*, **387,** 133–135.

Bohnert, S. and Schiavo, G. (2005). Tetanus toxin is transported in a novel neuronal compartment characterized by a specialized pH regulation. Submitted.

Bonifacino, J.S. and Glick, B.S. (2004). The mechanisms of vesicle budding and fusion. *Cell*, **116,** 153–166.

Boquet, P. and Duflot, E. (1982). Tetanus toxin fragment forms channels in lipid vesicles at low pH. *Proc. Natl. Acad. Sci. USA*, **79,** 7614–7618.

Borgese, N., Colombo, S. and Pedrazzini, E. (2003). The tale of tail-anchored proteins: coming from the cytosol and looking for a membrane. *J. Cell. Biol.*, **161,** 1013–1019.

Borowsky, B. and Hoffman, B.J. (1995). Neurotransmitter transporters: molecular biology, function, and regulation. *Int. Rev. Neurobiol.*, **38,** 139–199.

Brady, S.T., Pfister, K.K. and Bloom, G.S. (1990). A monoclonal antibody against kinesin inhibits both anterograde and retrograde fast axonal transport in squid axoplasm. *Proc. Natl. Acad. Sci. USA*, **87,** 1061–1065.

Breidenbach, M.A. and Brunger, A.T. (2004). Substrate recognition strategy for botulinum neurotoxin serotype A. *Nature*, **432,** 925–929.

Bridgman, P.C. (1999). Myosin Va movements in normal and dilute-lethal axons provide support for a dual filament motor complex. *J. Cell. Biol.*, **146,** 1045–1060.

Bychkova, V.E., Pain, R.H. and Ptitsyn, O.B. (1988). The "molten globule" state is involved in the translocation of proteins across membranes? *FEBS Lett.*, **238,** 231–234.

Cabiaux, V., Lorge, P., Vandenbranden, M., Falmagne, P. and Ruysschaert, J.M. (1985). Tetanus toxin induces fusion and aggregation of lipid vesicles containing phosphatidylinositol at low pH. *Biochem. Biophys. Res. Commun.*, **128,** 840–849.

Cajal, R.S. (1928). Studies on degeneration and regeneration of the nervous system.

Cao, T.T., Chang, W., Masters, S.E. and Mooseker, M.S. (2004). Myosin-Va binds to and mechanochemically couples microtubules to actin filaments. *Mol. Biol. Cell.*, **15,** 151–161.

Chan, W.K., Dickerson, A., Ortiz, D., Pimenta, A.F., Moran, C.M., Motil, J., Snyder, S.J., Malik, K., Pant, H.C. and Shea, T.B. (2004). Mitogen-activated protein kinase regulates neurofilament axonal transport. *J. Cell Sci.*, **117,** 4629–4642.

Chen, Y.A. and Scheller, R.H. (2001). SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell Biol.*, **2,** 98–106.

Coen, L., Kissa, K., le Mevel, S., Brulet, P. and Demeneix, B.A. (1999). A somatic gene transfer approach using recombinant fusion proteins to map muscle-motoneuron projections in Xenopus spinal cord. *Int. J. Dev. Biol.*, **43,** 823–830.

Coen, L., Osta, R., Maury, M. and Brulet, P. (1997). Construction of hybrid proteins that migrate retrogradely and transynaptically into the central nervous system. *Proc. Natl. Acad. Sci. USA*, **94,** 9400–9405.

Conner, S.D., Schmid, S.L., Masserini, M., Palestini, P. and Pitto, M. (2003). Regulated portals of entry into the cell. *Nature*, **422,** 37–44.

Cornille, F., Martin, L., Lenoir, C., Cussac, D., Roques, B.P. and Fournie-Zaluski, M.C. (1997). Cooperative exosite-dependent cleavage of synaptobrevin by tetanus toxin light chain. *J. Biol. Chem.*, **272,** 3459–3464.

Critchley, D.R., Nelson, P.G., Habig, W.H. and Fishman, P.H. (1985). Fate of tetanus toxin bound to the surface of primary neurons in culture: evidence for rapid internalization. *J. Cell. Biol.*, **100,** 1499–1507.

DasGupta, B.R. (1994). Structures of botulinum neurotoxin, its functional domains and perspectives on the crystalline type A toxin. In: *Therapy with Botulinum Toxin*. (eds. J. Jankovic and M. Hallett), pp. 15–39. Marcel Dekker, New York.

de Paiva, A., Poulain, B., Lawrence, G.W., Shone, C.C., Tauc, L. and Dolly, J.O. (1993). A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly. *J. Biol. Chem.*, **268,** 20838–20844.

Deak, F., Schoch, S., Liu, X., Sudhof, T.C. and Kavalali, E.T. (2004). Synaptobrevin is essential for fast synaptic-vesicle endocytosis. *Nat. Cell Biol.*, **6,** 1102–1108.

Dong, M., Richards, D.A., Goodnough, M.C., Tepp, W.H., Johnson, E.A. and Chapman, E.R. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol.*, **162,** 1293–1303.

Donovan, J.J. and Middlebrook, J.L. (1986). Ion-conducting channels produced by botulinum toxin in planar lipid membranes. *Biochemistry*, **25,** 2872–2876.

Duncan, M.J., Shin, J.S. and Abraham, S.N. (2002). Microbial entry through caveolae: variations on a theme. *Cell Microbiol.*, **4,** 783–791.

Evans, D.M., Williams, R.S., Shone, C.C., Hambleton, P., Melling, J. and Dolly, J.O. (1986). Botulinum neurotoxin type B. Its purification, radioiodination, and interaction with rat-brain synaptosomal membranes. *Eur. J. Biochem.*, **154,** 409–416.

Figueiredo, D.M., Hallewell, R.A., Chen, L.L., Fairweather, N.F., Dougan, G., Savitt, J.M., Parks, D.A. and Fishman, P.S. (1997). Delivery of recombinant tetanus-superoxide dismutase proteins to central nervous system neurons by retrograde axonal transport. *Exp. Neurol.*, **145,** 546–554.

Fishman, P.S., Savitt, J.M. and Farrand, D.A. (1990). Enhanced CNS uptake of systemically administered proteins through conjugation with tetanus C-fragment. *J. Neurol. Sci.*, **98,** 311–325.

Foran, P., Lawrence, G.W., Shone, C.C., Foster, K.A. and Dolly, J.O. (1996). Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. *Biochemistry*, **35,** 2630–2636.

Foran, P., Shone, C.C. and Dolly, J.O. (1994). Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry*, **33,** 15365–15374.

Francis, J.W., Bastia, E., Matthews, C.C., Parks, D.A., Schwarzschild, M.A., Brown, R.H., Jr. and Fishman, P.S. (2004b). Tetanus toxin fragment C as a vector to enhance delivery of proteins to the CNS. *Brain Res.*, **1011,** 7–13.

Francis, J.W., Figueiredo, D., vanderSpek, J.C., Ayala, L.M., Kim, Y.S., Remington, M.P., Young, P.J., Lorson, C.L., Ikebe, S., Fishman, P.S. and Brown, R.H., Jr. (2004a). A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. *Brain Res.*, **995,** 84–96.

Francis, J.W., Hosler, B.A., Brown, R.H., Jr. and Fishman, P.S. (1995). CuZn superoxide dismutase (SOD-1):tetanus toxin fragment C hybrid protein for targeted delivery of SOD-1 to neuronal cells. *J. Biol. Chem.*, **270,** 15434–15442.

Garcia, E.P., McPherson, P.S., Chilcote, T.J., Takei, K. and De Camilli, P. (1995). rbSec1A and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but are not in a stable complex with syntaxin. *J. Cell Biol.*, **129,** 105–120.

Geddes, J.W., Hess, E.J., Hart, R.A., Kesslak, J.P., Cotman, C.W. and Wilson, M.C. (1990). Lesions of hippocampal circuitry define synaptosomal-associated protein-25 (SNAP-25) as a novel presynaptic marker. *Neuroscience*, **38,** 515–525.

Gil, C., Chaib-Oukadour, I. and Aguilera, J. (2003). C-terminal fragment of tetanus toxin heavy chain activates Akt and MEK/ERK signaling pathways in a Trk receptor-dependent manner in cultured cortical neurons. *Biochem. J.*, **373,** 613–620.

Gil, C., Chaib-Oukadour, I., Blasi, J. and Aguilera, J. (2001). HC fragment (C-terminal portion of the heavy chain) of tetanus toxin activates protein kinase C isoforms and phosphoproteins involved in signal transduction. *Biochem. J.*, **356,** 97–103.

Gil, C., Ruiz-Meana, M., Alava, M., Yavin, E. and Aguilera, J. (1998). Tetanus toxin enhances protein kinase C activity translocation and increases polyphosphoinositide hydrolysis in rat cerebral cortex preparations. *J. Neurochem.*, **70,** 1636–1643.

Gill, D.M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.*, **46,** 86–94.

Goldstein, L.S. and Yang, Z. (2000). Microtubule-based transport systems in neurons: the roles of kinesins and dyneins. *Annu. Rev. Neurosci.*, **23,** 39–71.

Griffin, J.W. and Watson, D.F. (1988). Axonal transport in neurological disease. *Ann. Neurol.*, **23,** 3–13.

Guo, Z., Turner, C. and Castle, D. (1998). Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell*, **94,** 537–548.

Habermann, E. and Dreyer, F. (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. *Curr. Top Microbiol. Immunol.*, **129,** 93–179.

Habermann, E., Dreyer, F. and Bigalke, H. (1980). Tetanus toxin blocks the neuromuscular transmission *in vitro* like botulinum A toxin. *Naunyn. Schmiedebergs Arch. Pharmacol.*, **311,** 33–40.

Habermann, E. and Erdmann, G. (1978). Pharmacokinetic and histoautoradiographic evidence for the intraaxonal movement of toxin in the pathogenesis of tetanus. *Toxicon*, **16,** 611–623.

Hafezparast, M., Klocke, R., Ruhrberg, C., Marquardt, A., Ahmad-Annuar, A., Bowen, S., Lalli, G., Witherden, A.S., Hummerich, H., Nicholson, S. (2003). Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science*, **300,** 808–812.

Halpern, J.L. and Loftus, A. (1993). Characterization of the receptor-binding domain of tetanus toxin. *J. Biol. Chem.*, **268,** 11188–11192.

Halpern, J.L. and Neale, E.A. (1995). Neurospecific binding, internalization, and retrograde axonal transport. *Curr. Top Microbiol. Immunol.*, **195,** 221–241.

Hanson, M.A. and Stevens, R.C. (2000). Cocrystal structure of synaptobrevin-II bound to botulinum neurotoxin type B at 2.0 A resolution. *Nat. Struct. Biol.*, **7,** 687–692.

Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T. and Niemann, H. (1995). Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. *Embo. J.*, **14,** 2317–2325.

Helting, T.B. and Zwisler, O. (1977). Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J. Biol. Chem.*, **252,** 187–193.

Herreros, J., Lalli, G., Montecucco, C. and Schiavo, G. (2000b). Tetanus toxin fragment C binds to a protein present in neuronal cell lines and motoneurons. *J. Neurochem.*, **74,** 1941–1950.

Herreros, J., Lalli, G. and Schiavo, G. (2000a). C-terminal half of tetanus toxin fragment C is sufficient for neuronal binding and interaction with a putative protein receptor. *Biochem. J.*, **347,** 199–204.

Herreros, J., Ng, T. and Schiavo, G. (2001). Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. **12,** 2947–2960.

Hess, D.T., Slater, T.M., Wilson, M.C. and Skene, J.H. (1992). The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. *J. Neurosci.*, **12,** 4634– 4641.

Humeau, Y., Doussau, F., Grant, N.J. and Poulain, B. (2000). How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie.*, **82,** 427–446.

Hurd, D.D. and Saxton, W.M. (1996). Kinesin mutations cause motor neuron disease phenotypes by disrupting fast axonal transport in Drosophila. *Genetics*, **144,** 1075–1085.

Inoue, A. and Akagawa, K. (1992). Neuron-specific antigen HPC-1 from bovine brain reveals strong homology to epimorphin, an essential factor involved in epithelial morphogenesis: identification of a novel protein family. *Biochem. Biophys. Res. Commun.*, **187,** 1144–1150.

Inserte, J., Najib, A., Pelliccioni, P., Gil, C. and Aguilera, J. (1999). Inhibition by tetanus toxin of sodium-dependent, high-affinity [3H]5-hydroxytryptamine uptake in rat synaptosomes. *Biochem. Pharmacol.*, **57,** 111–120.

Jahn, R. and Sudhof, T.C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem.*, **68,** 863–911.

Jongeneel, C.V., Bouvier, J. and Bairoch, A. (1989). A unique signature identifies a family of zinc-dependent metallopeptidases. *FEBS Lett.*, **242,** 211–214.

Kahn, R.A., Fu, H. and Roy, C.R. (2002). Cellular hijacking: a common strategy for microbial infection. *Trends Biochem. Sci.*, **27,** 308–314.

Kamal, A. and Goldstein, L.S. (2000). Connecting vesicle transport to the cytoskeleton. *Curr. Opin. Cell Biol.*, **12,** 503–508.

Kee, Y., Lin, R.C., Hsu, S.C. and Scheller, R.H. (1995). Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron*, **14,** 991–998.

Keller, J.E., Cai, F. and Neale, E.A. (2004). Uptake of botulinum neurotoxin into cultured neurons. *Biochemistry*, **43,** 526–532.

Kemplay, S. and Cavanagh, J.B. (1983). Effects of acrylamide and botulinum toxin on horseradish peroxidase labeling of trigeminal motor neurons in the rat. *J. Anat.*, **137** (Pt 3), 477–482.

Kitamura, M., Takamiya, K., Aizawa, S. and Furukawa, K. (1999). Gangliosides are the binding substances in neural cells for tetanus and botulinum toxins in mice. *Biochim. Biophys. Acta*, **1441,** 1–3.

Koriazova, L.K. and Montal, M. (2003). Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat. Struct. Biol.*, **10,** 13–18.

Kraszewski, K., Mundigl, O., Daniell, L., Verderio, C., Matteoli, M. and De Camilli, P. (1995). Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. *J. Neurosci.*, **15,** 4328–4342.

Kristensson, K. and Olsson, T. (1978). Uptake and retrograde axonal transport of horseradish peroxidase in botulinum-intoxicated mice. *Brain Res.*, **155,** 118–123.

Kryzhanovsky, G.N. (1958). Central nervous changes in experimental tetanus and the mode of action of the tetanus toxin. Communication I. Irradiation of the excitation on stimulating the tetanized limb. *Bull. Exp. Biol. Med.*, **44,** 1456–1464.

Kurazono, H., Mochida, S., Binz, T., Eisel, U., Quanz, M., Grebenstein, O., Wernars, K., Poulain, B., Tauc, L. and Niemann, H. (1992). Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A. *J. Biol. Chem.*, **267,** 14721–14729.

Lacy, D.B., Tepp, W., Cohen, A.C., DasGupta, B.R. and Stevens, R.C. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat. Struct. Biol.*, **5,** 898–902.

Lalli, G. (2002b). The binding fragment of tetanus neurotoxin: A probe to study neuronal endocytosis and retrograde transport. *Biochemistry*. University College London, London.

Lalli, G., Bohnert, S., Deinhardt, K., Verastegui, C. and Schiavo, G. (2003b). The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol.*, **11,** 431–437.

Lalli, G., Gschmeissner, S. and Schiavo, G. (2003a). Myosin Va and microtubule-based motors are required for fast axonal retrograde transport of tetanus toxin in motor neurons. *J. Cell Sci.*, **116,** 4639–4650.

Lalli, G., Herreros, J., Osborne, S.L., Montecucco, C., Rossetto, O. and Schiavo, G. (1999). Functional characterization of tetanus and botulinum neurotoxins binding domains. *J. Cell Sci.*, **112** (Pt 16), 2715–2724.

Lalli, G. and Schiavo, G. (2002a). Analysis of retrograde transport in motor neurons reveals common endocytic carriers for tetanus toxin and neurotrophin receptor p75NTR. *J. Cell Biol.*, **156,** 233–239.

Li, L. and Singh, B.R. (1998). Isolation of synaptotagmin as a receptor for types A and E botulinum neurotoxin and analysis of their comparative binding using a new microtiter plate assay. *J. Nat. Toxins*, **7,** 215–226.

Lin, R.C. and Scheller, R.H. (1997). Structural organization of the synaptic exocytosis core complex. *Neuron*, **19,** 1087–1094.

Louch, H.A., Buczko, E.S., Woody, M.A., Venable, R.M. and Vann, W.F. (2002). Identification of a binding site for ganglioside on the receptor binding domain of tetanus toxin. *Biochemistry*, **41,** 13644–13652.

Mandelkow, E.M., Thies, E., Trinczek, B., Biernat, J. and Mandelkow, E. (2004). MARK/PAR1 kinase is a regulator of microtubule-dependent transport in axons. *J. Cell Biol.*, **167,** 99–110.

Martin, M., Iyadurai, S.J., Gassman, A., Gindhart, J.G., Jr., Hays, T.S. and Saxton, W.M. (1999). Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport. *Mol. Biol. Cell*, **10,** 3717–3728.

Maskos, U., Kissa, K., St Cloment, C. and Brulet, P. (2002). Retrograde trans-synaptic transfer of green fluorescent protein allows the genetic mapping of neuronal circuits in transgenic mice. *Proc. Natl. Acad. Sci. USA*, **99,** 10120–10125.

Matteoli, M., Takei, K., Perin, M.S., Sudhof, T.C. and De Camilli, P. (1992). Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.*, **117,** 849–861.

Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G. and Montecucco, C. (1996). Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proc. Natl. Acad. Sci. USA*, **93,** 13310–13315.

Menestrina, G., Forti, S. and Gambale, F. (1989). Interaction of tetanus toxin with lipid vesicles. Effects of pH, surface charge, and transmembrane potential on the kinetics of channel formation. *Biophys. J.*, **55,** 393–405.

Miana-Mena, F.J., Munoz, M.J., Ciriza, J., Soria, J., Brulet, P., Zaragoza, P. and Osta, R. (2003). Fragment C tetanus toxin: a putative activity-dependent neuroanatomical tracer. *Acta Neurobiol. Exp.* (*Wars*), **63,** 211–218.

Miana-Mena, F.J., Roux, S., Benichou, J.C., Osta, R. and Brulet, P. (2002). Neuronal activity-dependent membrane traffic at the neuromuscular junction. *Proc. Natl. Acad. Sci. USA*, **99,** 3234–3239.

Miesenbock, G., De Angelis, D.A. and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, **394,** 192–195.

Mochida, S., Poulain, B., Weller, U., Habermann, E. and Tauc, L. (1989). Light chain of tetanus toxin intracellularly inhibits acetylcholine release at neuro-neuronal synapses, and its internalization is mediated by heavy chain. *FEBS Lett.*, **253,** 47–51.

Montal, M.S., Blewitt, R., Tomich, J.M. and Montal, M. (1992). Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins. *FEBS Lett.*, **313,** 12–18.

Montecucco, C. (1986). How do tetanus and botulinum toxins bind to neuronal membranes. *Trends Biochem. Sci.*, **11,** 315–317.

Montecucco, C., Papini, E. and Schiavo, G. (1994). Bacterial protein toxins penetrate cells via a four-step mechanism. *FEBS Lett.*, **346,** 92–98.

Montecucco, C., Rossetto, O. and Schiavo, G. (2004). Presynaptic receptor arrays for clostridial neurotoxins. *Trends Microbiol.*, **12,** 442–446.

Montecucco, C., Schiavo, G. and Dasgupta, B.R. (1989). Effect of pH on the interaction of botulinum neurotoxins A, B, and E with liposomes. *Biochem. J.*, **259,** 47–53.

Montesano, R., Roth, J., Robert, A. and Orci, L. (1982). Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature*, **296,** 651–653.

Mundigl, O., Verderio, C., Krazewski, K., De Camilli, P. and Matteoli, M. (1995). A radioimmunoassay to monitor synaptic activity in hippocampal neurons in vitro. *Eur. J. Cell. Biol.*, **66,** 246–256.

Munro, P., Kojima, H., Dupont, J.L., Bossu, J.L., Poulain, B. and Boquet, P. (2001). High sensitivity of mouse neuronal cells to tetanus toxin requires a GPI-anchored protein. *Biochem. Biophys. Res. Commun.*, **289,** 623–629.

Murthy, V.N. and De Camilli, P. (2003). Cell biology of the presynaptic terminal. *Annu. Rev. Neurosci.*, **26,** 701–728.

Najib, A., Pelliccioni, P., Gil, C. and Aguilera, J. (1999). Clostridium neurotoxins influence serotonin uptake and release differently in rat brain synaptosomes. *J. Neurochem.*, **72,** 1991–1998.

Neubauer, V. and Helting, T.B. (1981). Structure of tetanus toxin: the arrangement of papain digestion products within the heavy chain-light chain framework of extracellular toxin. *Biochim. Biophys. Acta.*, **668,** 141–148.

Niemann, H. (1991). Molecular biology of clostridial neurotoxins. In: *A Sourcebook of Bacterial Protein Toxins*. (eds. J.E.A.a.J.H. Freer), pp. 303–348. Academic Press, London.

Nishiki, T., Tokuyama, Y., Kamata, Y., Nemoto, Y., Yoshida, A., Sato, K., Sekiguchi, M., Takahashi, M. and Kozaki, S. (1996). The high-affinity binding of Clostridium botulinum type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1a. *FEBS Lett.*, **378,** 253–257.

Oblatt-Montal, M., Yamazaki, M., Nelson, R. and Montal, M. (1995). Formation of ion channels in lipid bilayers by a peptide with the predicted transmembrane sequence of botulinum neurotoxin A. *Protein Sci.*, **4,** 1490–1497.

Osen-Sand, A., Catsicas, M., Staple, J.K., Jones, K.A., Ayala, G., Knowles, J., Grenningloh, G. and Catsicas, S. (1993). Inhibition of axonal growth by SNAP-25 antisense oligonucleotides *in vitro* and *in vivo*. *Nature*, **364,** 445–448.

Osen-Sand, A., Staple, J.K., Naldi, E., Schiavo, G., Rossetto, O., Petitpierre, S., Malgaroli, A., Montecucco, C. and Catsicas, S. (1996). Common and distinct fusion proteins in axonal growth and transmitter release. *J. Comp. Neurol.*, **367,** 222–234.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E. and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J. Cell. Biol.*, **109,** 3039–3052.

Parton, R.G., Ockleford, C.D. and Critchley, D.R. (1987). A study of the mechanism of internalization of tetanus toxin by primary mouse spinal cord cultures. *J. Neurochem.*, **49,** 1057–1068.

Pellizzari, R., Mason, S., Shone, C.C. and Montecucco, C. (1997). The interaction of synaptic vesicle-associated membrane protein/synaptobrevin with botulinum neurotoxins D and F. *FEBS Lett.*, **409,** 339–342.

Pellizzari, R., Rossetto, O., Lozzi, L., Giovedi, S., Johnson, E., Shone, C.C. and Montecucco, C. (1996). Structural determinants of the specificity for synaptic vesicle-associated membrane protein/synaptobrevin of tetanus and botulinum type B and G neurotoxins. *J. Biol. Chem.*, **271,** 20353–20358.

Penner, R., Neher, E. and Dreyer, F. (1986). Intracellularly injected tetanus toxin inhibits exocytosis in bovine adrenal chromaffin cells. *Nature*, **324,** 76–78.

Pike, L.J. (2003). Lipid rafts: bringing order to chaos. *J. Lipid Res.*, **44,** 655–667.

Ponomarev, A.W. (1928). Zur Frage der Pathogenese des Tetanus und des Fortbewegungsmechanismus des Tetanustoxin entlang der Nerven. *Z. Ges. Exp. Med.*, **61,** 93–106.

Poulain, B., Tauc, L., Maisey, E.A., Wadsworth, J.D., Mohan, P.M. and Dolly, J.O. (1988). Neurotransmitter release is blocked intracellularly by botulinum neurotoxin, and this requires uptake of both toxin polypeptides by a process mediated by the larger chain. *Proc. Natl. Acad. Sci. USA*, **85,** 4090–4094.

Qian, Y., Galli, A., Ramamoorthy, S., Risso, S., DeFelice, L.J. and Blakely, R.D. (1997). Protein kinase C activation regulates human serotonin transporters in HEK-293 cells via altered cell surface expression. *J. Neurosci.*, **17,** 45–57.

Rao, K.N., Kumaran, D., Binz, T. and Swaminathan, S. (2005). Structural analysis of the catalytic domain of tetanus neurotoxin. *Toxicon*, in press.

Ratts, R., Zeng, H., Berg, E.A., Blue, C., McComb, M.E., Costello, C.E., vanderSpek, J.C. and Murphy, J.R. (2003). The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. *J. Cell Biol.*, **160,** 1139–1150.

Rodger, J., Davis, S., Laroche, S., Mallet, J. and Hicks, A. (1998). Induction of long-term potentiation *in vivo* regulates alternate splicing to alter syntaxin 3 isoform expression in rat dentate gyrus. *J. Neurochem.*, **71,** 666–675.

Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R.H. and Montecucco, C. (1996). VAMP/synaptobrevin isoforms 1 and 2 are widely and differentially expressed in nonneuronal tissues. *J. Cell Biol.*, **132,** 167–179.

Rossetto, O., Schiavo, G., Montecucco, C., Poulain, B., Deloye, F., Lozzi, L. and Shone, C.C. (1994). SNARE motif and neurotoxins. *Nature*, **372,** 415–416.

Rossetto, O., Seveso, M., Caccin, P., Schiavo, G. and Montecucco, C. (2001). Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon*, **39,** 27–41.

Rossi, V., Picco, R., Vacca, M., D'Esposito, M., D'Urso, M., Galli, T. and Filippini, F. (2004). VAMP subfamilies identified by specific R-SNARE motifs. *Biol. Cell*, **96,** 251–256.

Rummel, A., Bade, S., Alves, J., Bigalke, H. and Binz, T. (2003). Two carbohydrate binding sites in the $H_{CC}$-domain of tetanus neurotoxin are required for toxicity. *J. Mol. Biol.*, **326,** 835–847.

Rummel, A., Karnath, T., Henke, T., Bigalke, H. and Binz, T. (2004a). Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J. Biol. Chem.*, **279,** 30865–30870.

Rummel, A., Mahrhold, S., Bigalke, H. and Binz, T. (2004b). The $H_{cc}$-domain of botulinum neurotoxins A and B exhibits a singular ganglioside binding site displaying serotype specific carbohydrate interaction. *Mol. Microbiol.*, **51,** 631–643.

Rupp, B. and Segelke, B. (2001). Questions about the structure of the botulinum neurotoxin B light chain in complex with a target peptide. *Nat. Struct. Biol.*, **8,** 663–664.

Salaun, C., James, D.J., Greaves, J. and Chamberlain, L.H. (2004). Plasma membrane targeting of exocytic SNARE proteins. *Biochim. Biophys. Acta*, **1693,** 81–89.

Satpute-Krishnan, P., DeGiorgis, J.A. and Bearer, E.L. (2003). Fast anterograde transport of herpes simplex virus: role for the amyloid precursor protein of alzheimer's disease. *Aging Cell*, **2,** 305–318.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R. and Montecucco, C. (1992c). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*, **359,** 832–835.

Schiavo, G., Boquet, P., Dasgupta, B.R. and Montecucco, C. (1990b). Membrane interactions of tetanus and botulinum neurotoxins: a photolabeling study with photoactivatable phospholipids. *J. Physiol.* (*Paris*), **84,** 180–187.

Schiavo, G., Malizio, C., Trimble, W.S., Polverino de Laureto, P., Milan, G., Sugiyama, H., Johnson, E.A. and Montecucco, C. (1994). Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. *J. Biol. Chem.*, **269,** 20213–20216.

Schiavo, G., Matteoli, M. and Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. *Physiol. Rev.*, **80,** 717–766.

Schiavo, G., Papini, E., Genna, G. and Montecucco, C. (1990a). An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. *Infect. Immun.*, **58,** 4136–4141.

Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L. and Montecucco, C. (1992a). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. *Embo. J.*, **11,** 3577–3583.

Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B.R., Benfenati, F. and Montecucco, C. (1993b). Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D, and E. *J. Biol. Chem.*, **268,** 23784–23787.

Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B.R. and Montecucco, C. (1992b). Botulinum neurotoxins are zinc proteins. *J. Biol. Chem.*, **267,** 23479–23483.

Schiavo, G., Santucci, A., Dasgupta, B.R., Mehta, P.P., Jontes, J., Benfenati, F., Wilson, M.C. and Montecucco, C. (1993c). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett.*, **335,** 99–103.

Schiavo, G., Shone, C.C., Bennett, M.K., Scheller, R.H. and Montecucco, C. (1995). Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.*, **270,** 10566–10570.

Schiavo, G., Shone, C.C., Rossetto, O., Alexander, F.C. and Montecucco, C. (1993a). Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J. Biol. Chem.*, **268,** 11516–11519.

Schiavo, G., Stenbeck, G., Rothman, J.E. and Sollner, T.H. (1997). Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc. Natl. Acad. Sci. USA*, **94,** 997–1001.

Schmid, M.F., Robinson, J.P. and DasGupta, B.R. (1993). Direct visualization of botulinum neurotoxin-induced channels in phospholipid vesicles. *Nature*, **364,** 827–830.

Schmitt, A., Dreyer, F. and John, C. (1981). At least three sequential steps are involved in the tetanus toxin-induced block of neuromuscular transmission. *Naunyn Schmiedebergs Arch. Pharmacol.*, **317,** 326–330.

Schnapp, B.J. and Reese, T.S. (1989). Dynein is the motor for retrograde axonal transport of organelles. *Proc. Natl. Acad. Sci. USA*, **86,** 1548–1552.

Schnapp, B.J., Reese, T.S. and Bechtold, R. (1992). Kinesin is bound with high affinity to squid axon organelles that move to the plus-end of microtubules. *J. Cell. Biol.*, **119,** 389–399.

Schneider, H., Groves, M., Muhle, C., Reynolds, P.N., Knight, A., Themis, M., Carvajal, J., Scaravilli, F., Curiel, D.T., Fairweather, N.F. and Coutelle, C. (2000). Retargeting of adenoviral vectors to neurons using the Hc fragment of tetanus toxin. *Gene Ther.*, **7,** 1584–1592.

Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Sudhof, T.C. and Kavalali, E.T. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science*, **294,** 1117–1122.

Schwab, M.H., Bartholomae, A., Heimrich, B., Feldmeyer, D., Druffel-Augustin, S., Goebbels, S., Naya, F.J., Zhao, S., Frotscher, M., Tsai, M.J. and Nave, K.A. (2000). Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate terminal granule cell differentiation in the hippocampus. *J. Neurosci.*, **20,** 3714–3724.

Shone, C.C., Hambleton, P. and Melling, J. (1987). A 50-kDa fragment from the NH2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles. *Eur. J. Biochem.*, **167,** 175–180.

Simpson, L.L. (1982). The interaction between aminoquinolines and presynaptically acting neurotoxins. *J. Pharmacol. Exp. Ther.*, **222,** 43–48.

Simpson, L.L. (2000). Identification of the characteristics that underlie botulinum toxin potency: implications for designing novel drugs. *Biochimie*, **82,** 943–953.

Simpson, L.L., Coffield, J.A. and Bakry, N. (1994). Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A2 neurotoxins. *J. Pharmacol. Exp. Ther.*, **269,** 256–262.

Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362,** 318–324.

Sorensen, J.B., Nagy, G., Varoqueaux, F., Nehring, R.B., Brose, N., Wilson, M.C. and Neher, E. (2003). Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell*, **114,** 75–86.

Stanley, E.F. (1997). The calcium channel and the organization of the presynaptic transmitter release face. *Trends. Neurosci.*, **20,** 404–409.

Stevens, R.C., Evenson, M.L., Tepp, W. and DasGupta, B.R. (1991). Crystallization and preliminary X-ray analysis of botulinum neurotoxin type A. *J. Mol. Biol.*, **222,** 877–880.

Stöckel, K., Schwab, M. and Thoenen, H. (1975). Comparison between the retrograde axonal transport of nerve growth factor and tetanus toxin in motor, sensory and adrenergic neurons. *Brain Res.*, **99,** 1–16.

Stoeckel, K., Schwab, M. and Thoenen, H. (1977). Role of gangliosides in the uptake and retrograde axonal transport of cholera and tetanus toxin as compared to nerve growth factor and wheat germ agglutinin. *Brain Res.*, **132,** 273–285.

Sudhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature*, **375,** 645–653.

Sukonpan, C., Oost, T., Goodnough, M., Tepp, W., Johnson, E.A. and Rich, D.H. (2004). Synthesis of substrates and inhibitors of botulinum neurotoxin type A metalloprotease. *J. Pept. Res.*, **63,** 181–193.

Sutton, R.B., Fasshauer, D., Jahn, R. and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*, **395,** 347–353.

Swaminathan, S. and Eswaramoorthy, S. (2000). Structural analysis of the catalytic and binding sites of Clostridium botulinum neurotoxin B. *Nat. Struct. Biol.*, **7,** 693–699.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H. and O'Kane, C.J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. *Neuron*, **14,** 341–351.

Teng, F.Y., Wang, Y. and Tang, B.L. (2001). The syntaxins. *Genome Biol.*, **2,** 3012.3011–3012.3017.

Tsui-Pierchala, B.A., Encinas, M., Milbrandt, J. and Johnson, E.M., Jr. (2002). Lipid rafts in neuronal signaling and function. *Trends Neurosci.*, **25,** 412–417.

Umland, T.C., Wingert, L.M., Swaminathan, S., Furey, W.F., Schmidt, J.J. and Sax, M. (1997). Structure of the receptor binding fragment $H_C$ of tetanus neurotoxin. *Nat. Struct. Biol.*, **4,** 788–792.

Vaidyanathan, V.V., Yoshino, K., Jahnz, M., Dorries, C., Bade, S., Nauenburg, S., Niemann, H. and Binz, T. (1999). Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains and amino acid residues controlling the formation of

enzyme-substrate complexes and cleavage. *J. Neurochem*., **72,** 327–337.

Vale, R.D. (2003). The molecular motor toolbox for intracellular transport. *Cell*, **112,** 467–480.

Vallee, R.B. and Bloom, G.S. (1991). Mechanisms of fast and slow axonal transport. *Annu. Rev. Neurosci*., **14,** 59–92.

Vallee, R.B., Shpetner, H.S. and Paschal, B.M. (1989). The role of dynein in retrograde axonal transport. *Trends Neurosci*., **12,** 66–70.

van der Goot, F.G., Gonzalez-Manas, J.M., Lakey, J.H. and Pattus, F. (1991). A "molten-globule" membrane-insertion intermediate of the pore-forming domain of colicin A. *Nature*, **354,** 408–410.

Veit, M., Sollner, T.H. and Rothman, J.E. (1996). Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25. *FEBS Lett*., **385,** 119–123.

Wagner, O.I., Ascano, J., Tokito, M., Leterrier, J.F., Janmey, P.A. and Holzbaur, E.L. (2004). The interaction of neurofilaments with the microtubule motor cytoplasmic dynein. *Mol. Biol. Cell*, **15,** 5092–5100.

Walch-Solimena, C., Blasi, J., Edelmann, L., Chapman, E.R., von Mollard, G.F. and Jahn, R. (1995). The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell Biol*., **128,** 637–645.

Waller, A.V. (1852). A new method for the study of the nervous system. *Lond. J. Med*., **43,** 609–625.

Washbourne, P., Pellizzari, R., Baldini, G., Wilson, M.C. and Montecucco, C. (1997). Botulinum neurotoxin types A and E require the SNARE motif in SNAP-25 for proteolysis. *FEBS Lett*., **418,** 1–5.

Weiss, P. and Hiscoe, H.B. (1948). Experiments on the mechanim of nerve growth. *J. Exp. Zool*., **107,** 315–395.

Weller, U., Dauzenroth, M.E., Gansel, M. and Dreyer, F. (1991). Cooperative action of the light chain of tetanus toxin and the heavy chain of botulinum toxin type A on the transmitter release of mammalian motor endplates. *Neurosci. Lett*., **122,** 132–134.

Weller, U., Dauzenroth, M.E., Meyer zu Heringdorf, D. and Habermann, E. (1989). Chains and fragments of tetanus toxin. Separation, reassociation and pharmacological properties. *Eur. J. Biochem*., **182,** 649–656.

Wellhoner, H.H., Hensel, B. and Seib, U.C. (1973). Local tetanus in cats: neuropharmacokinetics of 125 I-tetanus toxin. *Naunyn Schmiedebergs Arch. Pharmacol*., **276,** 375–386.

Whitmarsh, A.J. and Davis, R.J. (2001). Signal transduction by target-derived neurotrophins. *Nat. Neurosci*., **4,** 963–964.

Williamson, L.C., Bateman, K.E., Clifford, J.C. and Neale, E.A. (1999). Neuronal sensitivity to tetanus toxin requires gangliosides. *J. Biol. Chem*., **274,** 25173–25180.

Williamson, L.C., Halpern, J.L., Montecucco, C., Brown, J.E. and Neale, E.A. (1996). Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J. Biol. Chem*., **271,** 7694–7699.

Williamson, L.C. and Neale, E.A. (1994). Bafilomycin A1 inhibits the action of tetanus toxin in spinal cord neurons in cell culture. *J. Neurochem*., **63,** 2342–2345.

Wilson, M.C., Mehta, P.P. and Hess, E.J. (1996). SNAP-25, enSNAREd in neurotransmission and regulation of behaviour. *Biochem. Soc. Trans*., **24,** 670–676.

Wright, J.F., Pernollet, M., Reboul, A., Aude, C. and Colomb, M.G. (1992). Identification and partial characterization of a low affinity metal-binding site in the light chain of tetanus toxin. *J. Biol. Chem*., **267,** 9053–9058.

Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E.M., Sudhof, T.C. and Jahn, R. (1994a). Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. *J. Biol. Chem*., **269,** 12764–12772.

Yamasaki, S., Binz, T., Hayashi, T., Szabo, E., Yamasaki, N., Eklund, M., Jahn, R. and Niemann, H. (1994b). Botulinum neurotoxin type G proteolyses the Ala81-Ala82 bond of rat synaptobrevin 2. *Biochem. Biophys. Res. Commun*., **200,** 829–835.

Yamasaki, S., Hu, Y., Binz, T., Kalkuhl, A., Kurazono, H., Tamura, T., Jahn, R., Kandel, E. and Niemann, H. (1994c). Synaptobrevin/vesicle-associated membrane protein (VAMP) of *Aplysia californica*: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F. *Proc. Natl. Acad. Sci. USA*, **91,** 4688–4692.

Yowler, B.C., Kensinger, R.D. and Schengrund, C.L. (2002). Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. *J. Biol. Chem*., **277,** 32815–32819.

CHAPTER

# 21

# Large clostridial cytotoxins modifying small GTPases

*Maja Rupnik and Ingo Just*

## INTRODUCTION

Large clostridial cytotoxins (LCTs) are a family of toxins similar in origin, primary structure, enzymatic glycosyltransferase activity, and sharing sequence similarities ranging from 42 to 96%. They are protein exotoxins produced by clostridia and possess cytotoxic activity that is characterized by cell rounding and formation of transient arborized morphology based on disaggregation of the actin cytoskeleton. The LCTs are single-chained proteins (range from 250–308 kDa) and composed of three functional domains (Figure 21.1) responsible for cell entry and intracellular inactivation of small regulatory GTPases by covalent modification (Just *et al*., 2000; Busch and Aktories, 2000; Just and Gerhard, 2004). Their major targets are the Rho-GTPases and to a minor extent the Ras-GTPases. The Rho-GTPases are the master regulators of the actin cytoskeleton, but beyond the regulation of cytoskeleton-dependent cell functions, they are also involved in the regulation of transcription, apoptosis, and transformation.

The family encompasses toxin A (TcdA) and toxin B (TcdB) from *Clostridium difficile*, the lethal (TcsL) and the hemorrhagic toxin (TcsH) from *Clostridium sordellii*, and the α-toxin (TcnA) from *Clostridium novyi* (Bette *et al*., 1991; von Eichel-Streiber *et al*., 1996). *C. difficile* toxins show high similarity with *C. sordellii* toxins in biological activities, as well as in cross-reactivity with the same polyclonal and monoclonal antibodies (Martinez and Wilkins, 1992). TcdA is a functional homologue of TcsH, and TcdB of TcsL. TcnA is only distantly related to the remaining four toxins and does not cross-react with *C. difficile* or *C. sordellii* specific antibodies (Ball *et al*., 1993). Recently, a unified nomenclature of LCTs was agreed to and will be adopted also in this chapter (Rupnik *et al*., 2005a).

The producing microorganisms cause intestinal infections in humans and animals (*C. difficile* and *C. sordellii*) or gas gangrene (*C. sordellii* and *C. novyi*) (Kelly *et al*., 1994; Bartlett, 1994; Kelly and LaMont, 1998; Hatheway, 1990). LCTs are their major virulence factors and responsible for the onset of symptoms. However, although toxins possess comparable activity at a cellular level, they differ in their *in vivo* effects and exhibit biological action at those sites at which they are delivered by their producing microbes. In addition to their clinical relevance, LCTs are used as tools in cell biology to study the role of Rho and Ras.

## MOLECULAR BIOLOGY OF LARGE CLOSTRIDIAL TOXINS

### Toxin genes and pathogenicity locus PaLoc

Genes have been sequenced for four of the five members of LCT: TcdA, TcdB, TcsL, and TcnA, and the results have confirmed previously known immunological and functional similarities within the LCT group (von Eichel-Streiber and Sauerborn, 1990; von Eichel-Streiber *et al*., 1990; Dove *et al*., 1990; Johnson *et al*., 1990; von Eichel-Streiber *et al*., 1992; Green *et al*., 1995; Hofmann *et al*., 1995). LCT toxins share similarity from

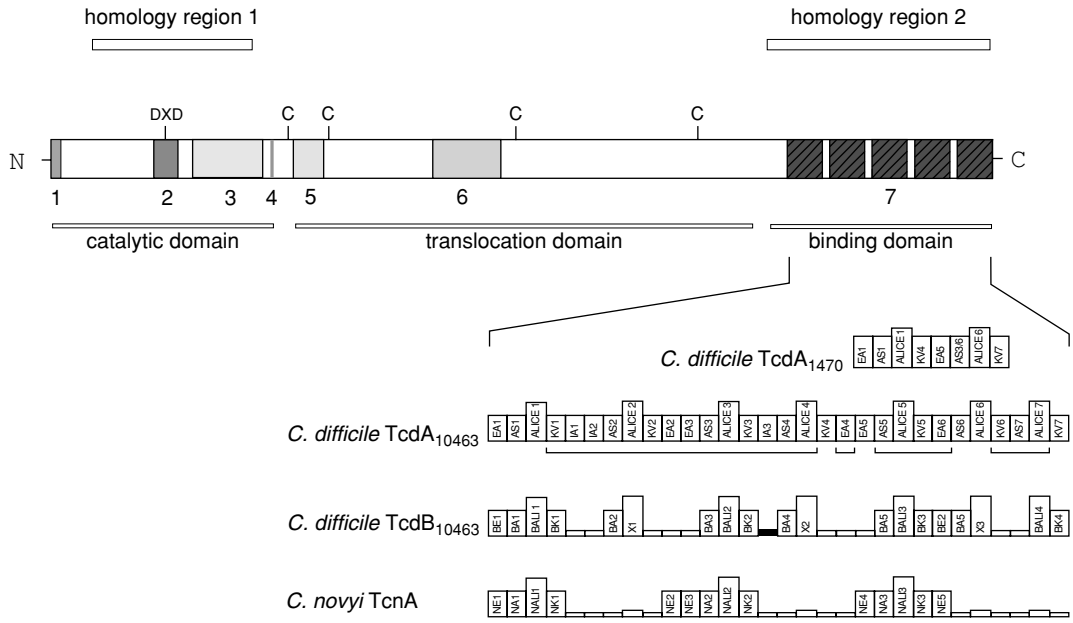



**FIGURE 21.1** Schematic presentation of a toxin from a LCT group. LCTs are large single-stranded proteins with three domains involved in enzymatic activity, translocation, and cell binding. The regions with known or putative functions are shown schematically and positions are given for TcdB (except for 1, which is described only in TcsL):
(1) binding site for phosphatidylserine (TcsL; aa 1 to 18);
(2) extended D-X-D motif (aa 264 to 296);
(3) GTPase specificity (aa 364–468, see text for discussion);
(4) cleavage site (aa 543/544);
(5) putative nucleotide (ATP) binding site (aa 651 to 683);
(6) highly hydrophobic region (aa 956 to 1128);
(7) repetitive sequences (from aa 1852 to 2366;)
DXD – glycosyltransferase motif; C – conserved cysteines (aa 595, 698, 1167, 1625)
Alignment of homologous repeats in TcdA, TcdB, and TcnA is presented. Repetitive domains are composed by 4 to 5 groups—long repeats have 49 to 52 aa (ALICE, BALI, NALI; X is a truncated form of BALI) and short repeats 20 aa (e.g., EA, AS, KV, and IA for TcdA). Additionally, deletions in $TcdA_{10463}$ (indicated by ???) leading to truncated repeats in $TcdA_{1470}$ in $A^-B^+$ toxinotype VIII are indicated.
Homology regions 1 and 2 mark two parts of LCTs that show amino acid sequence similarity with other microbial proteins (see text for discussion).

42 to 88% at amino acid sequence (Table 21.1); however, differences are mainly accumulated in the N-terminal catalytic domain, where the homology spans only from 28.8 to 78.6% (Thelestam *et al*., 1999; von Eichel-Streiber *et al*., 1995).

All LCT genes have similar structure with 3'-end repetitive regions coding for stretches of 20 to 52 amino acids, defined as clostridial repetitive oligopeptides, CROPs (von Eichel-Streiber *et al*., 1992), or repeating units (Dove *et al*., 1990) (Figure 21.1, Table 21.2). At the protein level in all four sequenced toxins, CROPs can be clustered according to the homology into four to five groups. Interestingly, the repeats are only conserved at the nucleotide level in the gene coding for TcdA, which results in frequent homologous recombinations resulting in deletions and insertions. A number of strains with shorter but still fully functional TcdA are described (Rupnik *et al*., 1998; Blake *et al*, 2004). No shorter variants of any other toxin from the LCT group have been reported so far.

TcdA, TcdB, TcsL, and TcsH are chromosomally encoded, whereas the gene for TcnA resides on a phage (Eklund, 1993). The toxin coding region is well characterized for TcdA and TcdB produced by *C. difficile*. It is defined as pathogenicity locus (PaLoc) and includes, besides two toxin genes *tcdA* and *tcdB*, three additional accessory genes (*tcdR, E,* and *C*) (Braun *et al*., 1996; Hammond and Johnson, 1995) (Figure 21.2). *tcdR* and *tcdC* are involved in the regulation of toxin production and will be discussed below. *tcdE* codes for a 170 aa hydrophobic protein homologous to phage holin protein, and was suggested to contribute to the release of the toxin from the bacterial cell (Tan *et al*., 2001).

PaLoc region is inserted in an identical integration site in several *C. difficile* strains tested (Braun *et al*., 1996; Hammond and Johnson, 1995). In non-toxigenic

**TABLE 21.1** Amino acid sequence similarity of LCT toxins

| | $TcdA_{10463}$ (X92928) | $TcdB_{10463}$ (X92928) | $TcdB_{1470}$ (Z23277) | $TcdB_{8864}$ (AJ011301) | $TcdL_{6018}$ (X82638) | TcnA (Z48636) | |
|---|---|---|---|---|---|---|---|
| $TcdA_{10463}$ | | 60 | 60 | 60 | 60 | 42 | |
| $TcdB_{10463}$ | 42 | | 96 | 93 | 87 | 48 | |
| $TcdB_{1470}$ | 41 | 93 | | 96 | 87 | 48 | **% similarity** |
| $TcdB_{8864}$ | 41 | 85 | 91 | | 88 | 47 | |
| $TcdL_{6018}$ | 41 | 75 | 75 | 75 | | 47 | |
| TcnA | 24 | 26 | 27 | 27 | 26 | | |
| | | | % identity | | | | |

GenBank Acc. numbers used are given in parentheses. Amino acid sequences were aligned with ClustalW, and identity and similarity was calculated in GeneDoc software (Blosum62).

**TABLE 21.2** Properties of LCT toxins

| | Toxin gene | | Protein | | | |
|---|---|---|---|---|---|---|
| toxin | length (bp) | location | size (kDa) | number of repeats | CPE[a] | target GTPases[b] |
| $TcdA_{10463}$ | 8130 | chromosome (PaLoc) | 308 | 30 | D | Rho, Rac, Cdc42, RhoG, TC10, (Rap) |
| $TcdA_{C34}$ | nd | nd | 308 | nd | D | Rho, Rac, Cdc42, Rap |
| $TcdB_{10463}$ | 7101 | chromosome (PaLoc) | 270 | 19 | D | Rho, Rac, Cdc42, RhoG, TC10 |
| $TcdB_{1470}$ | 7104 | chromosome (PaLoc) | 270 | 19 | S | Rac, Cdc42, Rap, Ral, R-Ras |
| $TcdB_{8864}$ | 7104 | chromosome (PaLoc) | 270 | 19 | S | Rac, Cdc42, Rap,Ral, R-Ras |
| $TcdB_{C34}$ | nd | nd | 270 | nd | S | Rho, Rac, Cdc42, Rap, Ral |
| $TcsH_{9048}$ | nd | nd | 308 | nd | nd | Rho, Rac, Cdc42 |
| $TcsL_{6018}$ | 7095 | chromosome | 270 | 19 | S | Rac, (Cdc42), RhoG, TC10, Ras, Ral, Rap |
| $TcsL_{1522}$ | nd | nd | 270 | nd | S | Rac, Ral, Rap, Ras |
| $TcsL_{9048}$ | nd | nd | 270 | nd | S | Rac, Cdc42, Rap, Ras |
| TcnA | 6534 | phage | 250 | 14 | D | Rho, Rac, Cdc42 |

[a]D: *C. difficile*-like, S: *S. sordellii*-like ; () -minor substrate

[b]adapted from references: Just *et al*., 1995a,b and 1996 and 2004; Hofmann *et al*., 1996; Popoff *et al*., 1996; Chaves-Olarte *et al*., 1997; Chaves-Olarte *et al*. 1999; Genth *et al*., 1996; Selzer *et al*., 1996

strains PaLoc is substituted by 115 bp non-coding sequence. The border sequences are perfectly conserved in toxinogenic as well as non-toxinogenic strains, indicating that PaLoc is a distinct genetic element. However, PaLoc itself does not show any evidence of being a mobile element, as there is no homology with transposon-, phage-, or plasmid-like sequences, no typical direct or inverted repeats, and no tRNA sequences either within or near the PaLoc (Braun *et al*., 1996). Still, the possibility that PaLoc is a part of an even larger virulence-associated element was not excluded.

Toxin coding region is less well characterized in *C. sordellii* and *C. novyi*. In both species partial sequences of regions near the toxin genes revealed the presence of small ORFs (Figure 21.2C). In *C. sordellii,* sequence is known only for ORF downstream of *tcsL*, and it shows 73% identity and 93% similarity to *tcdE* (Green *et al*., 1995). Interestingly, the toxin coding region was sequenced in variant *C. sordellii* strain 6018, which does not produce TcsH (Green *et al*., 1996). *C. difficile* strains not producing TcdA, which is a structural and functional homologue of TcsH, are widespread, but they always have at least a truncated *tcdA* gene. In *C. sordellii* 6018, on the other hand, no *tcsH* sequences could be detected by PCR or hybridization (Green *et al*., 1996). In *C. novyi* no sequence data is available, but orientation of two small ORFs upstream and downstream of *tcnA* correspond to *tcdR*/*tcdE* and *tcdC* (von Eichel-Streiber *et al*., 1994).

## Genetic manipulations of toxin coding and associated PaLoc regions

Only a few methods have been developed for DNA manipulation of LCT-producing clostridia, and they are generally available only for *C. difficile*. For this reason, heterologous hosts were predominately used for the expression of recombinant holotoxins or their functional domains (Hofmann *et al*., 1997; Wagenknecht-Wiesner *et al*., 1996; Frisch *et al*., 2003; Faust *et al*., 1998; Kink and Williams, 1998; Burger *et al*., 2003; Ackermann *et al*., 2004) and regulation studies

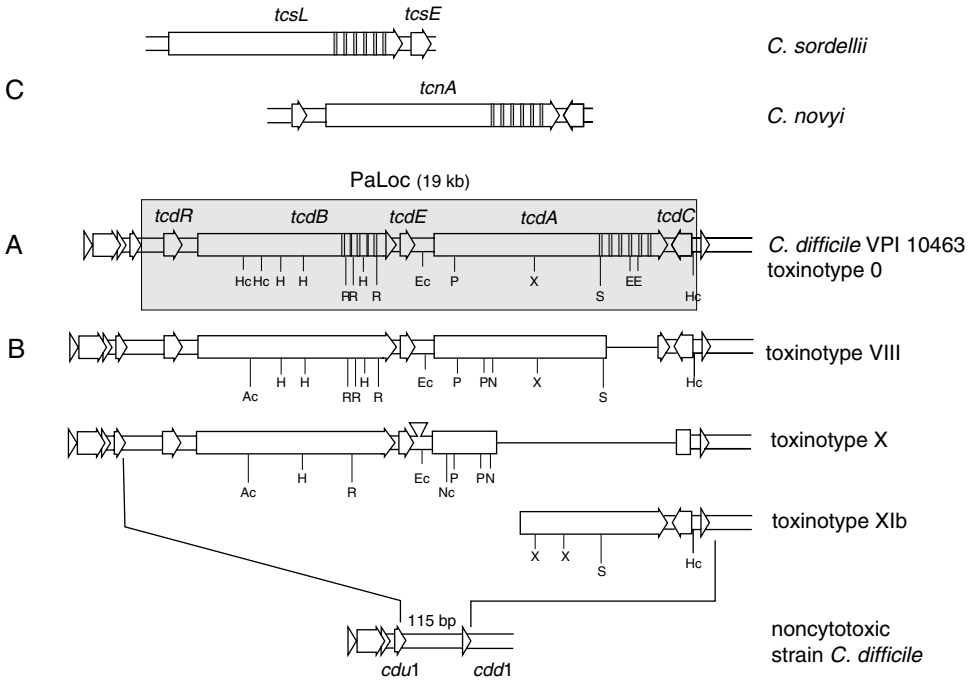



**FIGURE 21.2** Regions encoding the LCT toxins. **(A)** *C. difficile* pathogenicity locus PaLoc with two toxin genes, *tcdA* and *tcdB*, and three accessory genes, *tcdR, E, C*. Repetitive regions at 3'-end are hatched. Several restriction sites used in RFLPs analysis of variant strains are represented (Hc-*Hin*cII, H-*Hin*dIII, R-*Rsa*I, Ec-*Eco*RV, P-*Pst*I, X-*Xba*I, S-*Spe*I, E-*Eco*RI, Ac-*Acc*I, Nc-*Nco*I). **(B)** Variant PaLoc regions of some representative toxinotypes; note insertions (toxinotype X), changed restriction sites, and deletions in *tcdA*. In toxinotype XI only remnants of PaLoc are present and no functional LCT toxins are produced. In non-toxinogenic strains, PaLoc is replaced by 115 bp noncoding sequence. **(C)** Known toxin coding regions in *C. sordellii* and *C. novyi*; for *C. novyi* only position and orientation of two small ORFs is described in the literature, but no sequence data is available. Therefore, no similarities could be determined and ORFs are not named in this figure.

(see below), whereas only a few reports use *C. difficile* as a host (Tang-Feldman *et al*., 2002; Mani *et al*., 2002). For a review on early partial cloning and sequencing studies of the LCT toxin genes, the reader is referred to Moncrief and Wilkins (2000).

The complete *C. difficile* toxin genes were cloned and expressed in two different heterologous hosts: in *E. coli* (*tcdA:* Phelps *et al*., 1991; Ackermann *et al*., 2004; *tcdB*: Johnson *et al*., 1990) and *Bacillus megaterium* (*tcdA:* Burger *et al*., 2003). Expression in *E. coli* is very low, probably due to the different codon usage. Only the expression of TcdB (under its own promotor) was sufficient to allow mutagenesis studies (Barroso *et al*., 1994). The *B. megaterium* system resulted in a better yield of TcdA and enables a simple purification via His-tag (Burger *et al*., 2003). Tang-Feldman and co-workers (2002) have introduced a complete *tcdB* gene in non-toxigenic *C. difficile* strain; however, the transformation efficiency and the expression were again low and neither recombinant toxin nor the strain were used in further studies.

## Variability of LCT toxins and toxin coding regions

Variant toxins from *C. difficile* TcdB and *C. sordellii* TcsL with slight differences in substrate pattern are reported (Table 21.2), suggesting the divergence and variability in toxin coding regions. While variability in *C. novyi* is undocumented and in *C. sordellii* limited to a few examples (Green *et al*., 1996; Popoff *et al*., 1996; Hofmann *et al*., 1996), it is well recognized in *C. difficile* at the gene as well as at the protein level.

Toxin coding PaLoc region in *C. difficile* can display deletions, insertions, and numerous point mutations, resulting in changed RFLP patterns when compared to the reference strain VPI 10463 (Figure 21.2B). Such strains are defined as variant strains and are grouped into toxinotypes (Rupnik *et al*., 1998). Currently, 24 variant toxinotypes are described (I to XXIV), including about 10% of *C. difficile* strains, whereas strains identical to the reference VPI 10463 represent the most numerous toxinotype 0 (http://www.mf.uni-mb.si/mikro/tox/).

Deletions of various sizes are mostly limited to the repetitive regions of *tcdA*. Only in two toxinotypes, X and XI, is PaLoc significantly changed (Figure 21.2B). Gene *tcdC* was shown to frequently possess small deletions and nonsense mutations, but the possible modified function of truncated TcdC was not studied (Soehn *et al*., 1998; Spigaglia and Mastrantonio, 2002). Insertions in PaLoc are rare, and two examples include a 1.1 kb between *tcdE* and *tcdA* in strain 8864 (Figure 21.2B, toxinotype X) (Soehn *et al*., 1998; Song *et al*, 1999) and 2 kb in *tcdA* gene in strains of toxinotype XIV (Mehlig *et al*., 2001; Rupnik *et al*., 2001). The later insertion is a novel mobile genetic element, ISTron, which is precisely excised from mRNA. Therefore, TcdA produced by toxinotype XIV strains is of expected size and fully functional (Braun *et al*., 2000; Mehlig *et al*., 2001). Modified restriction sites are found throughout the PaLoc region, but they are predominate in the *tcdB* gene, while the *tcdA* gene seems to be more conserved (Rupnik *et al*., 1998).

The majority of variant toxinotypes produce both toxins ($A^+B^+$ strains), though variant $A^-B^+$ and $A^-B^-$ strains are also described. In fact, two groups of $A^-B^+$ *C. difficile* strains were the first variants to be recognized (Torres, 1991; Lyerly *et al*., 1992; Borriello *et al*, 1992; Depitre *et al*., 1993). The number is raised to five currently differentiated $A^-B^+$ toxinotypes (Johnson *et al*., 2003; Rupnik *et al*. 2003); however, the molecular mechanism underlying the lack of TcdA production is clarified only in two of them. In toxinotype VIII, it is due to a point mutation introducing a stop codon at amino acid position 47 of TcdA (von Eichel-Streiber *et al*., 1999). In toxinotype X, stop codon is introduced at amino acid position 699, but additional deletion and insertion in $PaLoc_{8864}$ both result in low levels of transcription of remaining *tcdA* and *tcdC*, making both gene products undetectable at the protein level (Soehn *et al*., 1998). Another representative group of strains with significantly changed PaLoc leading even to the absence of production of both toxins is toxinotype XI ($A^-B^-$), where only the remnants of whole length PaLoc are present (Rupnik *et al*., 2001) (Figure 21.2B).

Variations at the nucleotide level can result in production of LCT toxins with changed properties, such as immunoreactivity with different antibodies, minimal differences in size, different substrate patterns, and different cytopathic effects (Figure 21.8, Table 21.2). Only a couple of studies have described properties of variant toxins of $A^+B^+$ strains (Mehlig *et al*., 2001; Blake *et al*., 2003). Probably for historical reasons, the variant TcdBs produced by $A^-B^+$ strains, $TcdB_{1470}$ and $TcdB_{8864}$, are best characterized at the molecular and biochemical levels (Torres, 1991; von Eichel-Streiber *et al*., 1995; Rupnik *et al*., 1997; Soehn *et al*., 1998; Sambol *et al*., 2000; Kato *et al*., 1999; Chaves-Olarte *et al*., 1999). Unlike reference $TcdB_{10463}$, $TcdB_{8864}$ was reported to exhibit enterotoxic activity (Borriello *et al*., 1992) and had a lower lethal dose, similar to TcsL (6 ng; Lyerly *et al*., 1992). $TcdB_{8864}$ also showed minimal immunological differences and did not react with two mAbs specific for wild-type TcdB (Lyerly *et al*., 1992). $TcdB_{1470}$, the representative of the most widespread $A^-B^+$ group (toxinotype VIII), binds to the same receptors as $TcdB_{10463}$, but modifies similar targets as *C. sordellii* $TcsL_{1522}$ and was therefore postulated to be a hybrid toxin (Chaves-Olarte *et al*., 1999). $tcdB_{8864}$ and $tcdB_{1470}$ are similar to each other, but significantly different from $tcdB_{10463}$ in regions coding for catalytic domain (von Eichel-Streiber *et al*., 1995; Rupnik *et al*., 1997; Soehn *et al*., 1998). Correspondingly, both variant toxins differ from $TcdB_{10463}$ in recognizing GTPases and, as a consequence, in CPE (Chaves-Olarte *et al*., 1999; Chaves-Olarte *et al*., 2003) (Table 21.2, Figure 21.8). In addition to the two $A^-B^+$ toxinotypes mentioned, the same sordellii-like CPE is detected by several $A^+B^+$ toxinotypes (IX, XIV, XV, XVII) and is caused by variant TcdBs rather than by variant TcdAs (Blake *et al*., 2004; Rupnik *et al*., 2003).

In receptor binding repetitive regions, on the other hand, $tcdB_{8864}$ and $tcdB_{1470}$ differ one from another; $tcdB_{1470}$ is identical with the reference strain, whereas $tcdB_{8864}$ resembles some other variant strains (von Eichel-Streiber *et al*., 1995; Rupnik *et al*., 2001). No comparative studies on binding of both toxins were done, but similar changes at 3'-end of genes resulted in changed properties of the catalytic domain, and different properties of variant binding domains could be expected.

## Regulation of toxin production in C. *difficile*

Toxin production varies greatly among different toxigenic strains and is additionally influenced by culture conditions (temperature, atmosphere), as well as by the growth medium used (complex vs. defined medium, nutrients, addition of antibiotics). In all tested strains, toxin levels are up-regulated in complex glucose-deprived media but not in defined glucose-deprived media (Dupuy and Sonenshein, 1998, Karlsson *et al.*, 1999). Toxin levels are also up-regulated during biotin starvation (Yamakawa *et al.*, 1996), while the presence of cysteine and proline down-regulate the production (Karlsson *et al.*, 2000). The reports on the effect of other amino acids and sub-inhibitory levels of antibiotics are somewhat controversial, and seem to be strain and medium dependent (Onderdonk *et al*., 1979; Haslam *et al*. 1986; Barc *et al*., 1992; Osgood *et al*., 1993; Yamakawa *et al*., 1994; Karlsson *et al*., 1999; Drummond *et al*., 2003). Temperature regulation is observed only in

some tested strains, such as VPI 10463 and serogroup H, but not in serogroup A and C; however, temperature regulation is not medium dependent (Karlsson *et al*., 2003).

Regulation of toxin production at the molecular level is only partially understood, but recent advances in *C. difficile* genetics should facilitate further studies. Transcriptional studies have shown PaLoc genes to be organized in two distinct transcription units (Figure 21.3). Genes *tcdR, B, E, A* are found on polycistronic and monocystronic transcripts, and their expression is induced at the transition from the exponential to stationary phase (Hundsberger *et al*., 1997). *tcdA* and *tcdB* mRNAs reach their maximum values in the early stationary phase. At this stage, levels of *tcdA* mRNA are approximately twofold in comparison to *tcdB* (Dupuy and Sonenshein, 1998), which is in agreement with larger amounts of TcdA obtained after toxin purification (von Eichel-Streiber *et al*., 1987).

So far, two PaLoc accessory genes, *tcdC* and *tcdR* (also *tcdD* or *txeR*), are implicated in regulation. The role of TcdC as a negative regulator is based on the observation that transcription of *tcdR, B, E, A* is low during the high-level transcription of *tcdC* (Hundsberger *et al*., 1997). Moreover, TcdC specifically binds to TcdR, responsible for positive regulation (see below), and thereby decreases transcription from toxin promoters *in vitro* (B. Dupuy, personal communication).

TcdR (previously TcdD or TxeR), a 22 kDa protein, strongly activates expression from *C. difficile* toxin gene promoters when expressed in *trans* in *C. perfringens* (Mani and Dupuy, 2001) and *E. coli* (Moncrief *et al*., 1997) and was therefore suggested to be a positive regulator. However, Mani and Dupuy (2001) have shown that isolated TcdR acts by interacting with RNA polymerase core enzyme to stimulate its binding to the promoter region of *tcdA*, *tcdB* (Mani and Dupuy, 2001), and of *tcdR* (Mani *et al*., 2002) and therefore functions as an alternative sigma factor.

TcdR forms, together with TetR (regulation of tetanus toxin gene in *C. tetani*), BotR (regulation of BoNT in *C. botulinum*), and UviA (regulation of bacteriocin gene in *C. perfringens*) a new alternative sigma factor subgroup within $\sigma^{70}$ family (Mani and Dupuy, 2001; Raffestin *et al*., 2005; Dupuy *et al*., 2005). This new group is likely to represent a common regulatory mechanism for clostridial toxins and bacteriocins. All four regulators show high sequence similarity and are able to substitute for each other *in vitro* and *in vivo* (Dupuy). This functional complementarity correlates with a high conservation of -35 sequences of the promoters recognized by all four factors (Hundsberger *et al*., 1997; Hammond *et al*., 1997; Dupuy and Sonenshein, 1998; Raffestin *et al*., 2005).

TcdR mediates, at least partially, the temperature and glucose control of *C. difficile* toxin production (Mani *et al*., 2002; Karlsson *et al*., 2003). The presence of *tcdR* in *trans* increased expression from both toxin promoters at 37°C, but not at 22 or 42°C in *C. perfingens* system (Karlsson *et al*., 2003). In contrast, early studies on environmental effects on *Clostridium difficile* reported

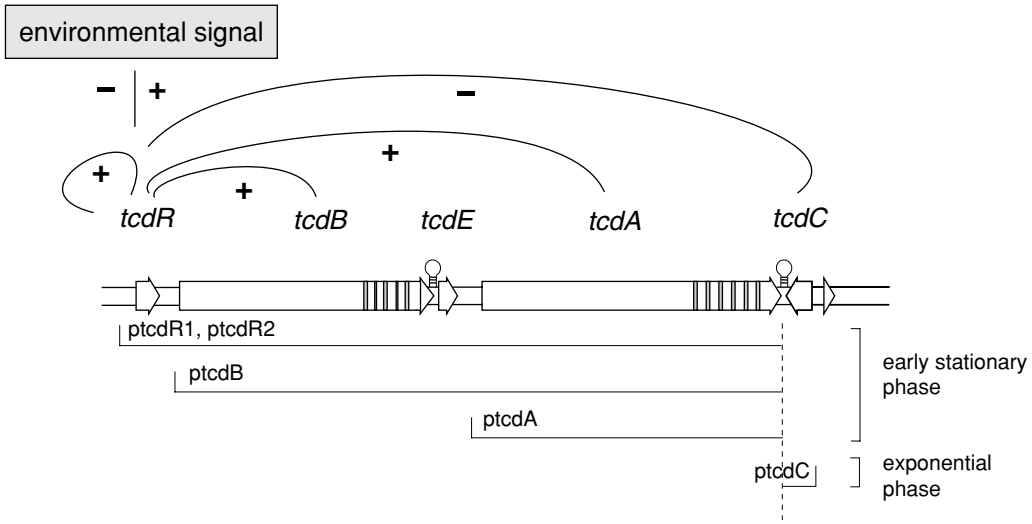



**FIGURE 21.3** Model for regulation of *C. difficile* toxin gene expression. The five PaLoc genes are transcribed in two discrete units: *tcdC* in exponential phase and *tcdR, B, E, A* as monocistronic or polycistronic mRNAs in stationary phase. Each PaLoc gene has own promoter (ptcd); however, transcription from *ptcdE* is not well documented. TcdR is transcribed from two promoters, which differ in sensitivity for the glucose. TcdR is a sigma factor and positively affects the transcription of itself and of both toxin genes. Environmental factors (e.g., temperature and glucose) act on TcdR transcription but could probably act also directly on the toxin genes.

increased toxin levels at 45°C in continous culture (Onderdoonk *et al*., 1979). During the studies of glucose effect on TcdR expression, it was discovered that *tcdR* gene has at least two promoters. One is glucose sensitive and is repressed by 1% glucose when tested in *C. perfringens* system (Mani *et al*., 2002). The second *tcdR* promoter is relatively weak, independent of TcdR, and insensitive on glucose, and provides a basal level of TcdR expression (Mani *et al*. 2002). Glucose could also act independently with the toxin promoters or via other regulatory mechanisms. However, the intermediate steps between environmental stimulus and transcription of PaLoc genes, including two component sensor/regulator systems or catabolite regulator protein (CcpA), remains largely unknown.

Unlike some other clostridial toxin genes (*C. perfringens* enterotoxin; Zhao and Melville, 1998), the production of *C. difficile* toxins A and B does not seem to be linked to sporulation (Kamiya *et al*., 1992). However, several other *C. difficile* proteins, such as enzymes involved in butyric acid production, electron carriers in oxidation-reduction reactions, and enzymes from purine biosythesis pathway are co-regulated with the toxin production in *C. difficile* (Karlsson *et al*., 2000; Maegawa *et al*., 2002; Karlsson *et al*., 2003).

Little is known about *in vivo* toxin expression and physiological relevance of toxin regulation. Glucose-dependent regulation could be an adaptation to the gut environment, as levels of free glucose are low in the large intestine (Karlsson *et al*., 2000). Association between amino acid limitation and increased toxin production may be of clinical relevance because protein malnutrition may be a risk factor for *C.Difficile*-associated disease (Karlsson *et al*., 2000).

## Related proteins from other organisms

There are several descriptions of a single toxin or highly conserved toxins being produced by different species, e.g., Shiga-like toxins or BoNTs. So far, only one report has described isolation of yet another clostridial species, *C. baratii*, producing a *C. difficile*-like toxin(s) (Ravizzola *et al*., 1998). The strain is no longer available, but the culture supernatant was cytotoxic and reacted with *C. difficile* commercial A/B kit.

However, LCTs show similarity with many other proteins, mainly putative glycosyltransferases. Interestingly, the C-terminal repeats and N-terminal enzymatic domain show homology with two different groups of proteins (Figure 21.1), suggesting a modular evolution of LCTs.

The C-terminal domain is similar to a number of nonrelated proteins with a common feature of binding saccharides, choline, or glucans (Wren, 1991): glycosyltransferases from oral streptococci *S. mutans* and *S. downei* and to lytic enzymes (amidases and muramidases) from *S. pneumoniae* and its bacteriophages (Wren, 1991; von Eichel-Streiber *et al*., 1992). In both *S. mutans* and *S. downei*, glycosyltransferases are extracellular proteins and are considered as virulence factors, contributing to dental plaque and caries formation (Wren, 1991).

The 500 to 700 aa N-terminal part of LCTs with glycosyltransferse D-X-D motif exhibit sequence similarity to gycosyltransferases involved in capsular polysaccharide synthesis, yeast OCH1 glycosyltransferases (Busch *et al*., 2000), and adhesion molecules. Most interesting, however, is the sequence homology of LCTs and several large putative toxic molecules, of which only one (LifA) was invariably linked with toxic action.

The homology to large plasmid-encoded protein in enterohemorrhagic *E. coli* (EHEC) was reported in 1998 (Burland *et al*., 1998), and the gene was designated *toxB* solely on the basis of similarity to LCTs. In addition to *toxB*, two other *E. coli* proteins are similar to *C. difficile* and *C. sordellii* toxins: Efa1 produced by some EHEC strains and LifA produced by enteropathogenic (EPEC) strains. LifA and Efa1 (both 3223 aa) are 97.4% identical to each other and 28% identical to *E. coli* ToxB. No enzymatic activity has yet been ascribed to ToxB, Efa1, and LifA (Stevens *et al*., 2004). Only LifA (lymphostatin) so far has been shown to have toxic properties. It inhibits lymphocyte proliferation and synthesis of proinflamatory cytokines, but unlike LCTs, does not cause changes in the cytoskeleton of HEp-2 cells (Klapproth *et al*., 2000).

Proteins similar to LCTs and Efa1/LifA were detected also in genomes of *Chlamidophyla caviae* and *C. muridarum*, whereas *C. trachomatis* possess only a truncated gene (Read *et al*., 2003). Both former species cause cytopathic effects on cells, but the mechanism is unclear. Putative toxins from *Chlamydophyla* and *Chlamydia* contain, besides LCT-like catalytic domain, also cysteine protease domain that could mediate proteolytic C-terminal cleavage of Rho (Shao *et al*., 2002). Other large uncharacterized proteins similar to LCTs are found in *C. acetobutylicum* and *P. fluorescens*.

# STRUCTURAL AND FUNCTIONAL PROPERTIES OF LCTS

LCTs are large, single-chained proteins with postulated three domain structure. Several functional motifs are recognized (Figure 21.1), whereby three of them are highly conserved and correlate with the three

functional domains: (i) D-X-D glycosytransferase motif within the catalytic domain, (ii) stretch of hydrophobic amino acids in putative translocation domain, and (iii) repetitive sequences forming the receptor binding domain.

All LCTs are cytotoxic and lethal, but only TcdA, TcdB, and TcsH possess enterotoxic activity. Cytotoxicity is primarily due to the glucosylation of Rho GTPases and subsequent effects on cell cytoskeleton and signaling pathways, whereas systemic effects of the toxins, such as lethality and enterotoxicity, are less understood.

## Cell entry

The LCTs act intracellularly and get access to their targets by receptor-mediated endocytosis and translocation to the cytoplasm from the endosomal compartment.

### *Receptor binding domain and cellular toxin receptor*

The first step of cell entry is the interaction with membranous structures to allow binding, which is thought to be mediated by the repetitive peptide elements (CROPs: combined repetitive oligopetides) located at the C-terminal part of each cytotoxin. The best studied prototype in this respect is TcdA. The previously discussed homology of repetitive peptide elements (about one-third of the TcdA molecule) with the carbohydrate-binding regions of glycosyltransferases from *Streptococcus mutans* (Von Eichel-Streiber and Sauerborn, 1990; Von Eichel-Streiber *et al*., 1992) indicates a structural hint that TcdA binds to cellular carbohydrate structures. This notion has been at least partially supported by various findings showing that treatment of cells with glycosidases, N-glycosylation inhibitor tunicamycin, or proteases reduce but do not abolish TcdA effects on cells (Pothoulakis *et al*., 1991; Pothoulakis *et al*., 1996; Smith *et al*., 1997). Furthermore, TcdA was shown to bind directly to the terminal carbohydrate structure Galα1-3Galβ1-4GlcNAc (Krivan *et al*., 1986; Tucker and Wilkins, 1991); however, this oligosaccharide is absent in humans (Larsen *et al*., 1990). Humans do have GalNAcβ1-3Galβ1-4GlcNAc-structure, to which TcdA also binds (Karlsson, 1995; Teneberg *et al*., 1996). The membranous sucrase-isomaltase glycoprotein was identified as a functional TcdA receptor in rabbit ileal brush border, but again, this receptor is not expressed in many toxin-sensitive tissues (e.g., human colon) (Pothoulakis *et al*., 1996). It is conceivable that TcdA recruits different receptors that are similar but not identical. The carbohydrate structure (containing at least Galβ1-4GlcN) seems to be the essential element for binding of TcdA to its cell receptor, but whether this carbohydrate is linked to proteins or lipids is unknown.

So far there are no data on the receptors of the other LCTs. Binding of TcsL to phosphatidylserine was reported for TcsL and the binding site is located at N-terminus (Mesmin *et al*., 2004) (Figure 21.1). However, phosphatidylserine is enriched on the cytoplasmic site of cell membranes, and this binding is thought to play a role in the docking of the catalytic fragment close to its membrane-associated GTPases substrate, rather than in the binding step of holotoxin.

That the C-terminal part of TcdA is, in fact, the receptor binding domain has been directly shown by competition studies testing the ability of different TcdA subfragments to block the cellular uptake of the holotoxin (Frisch *et al*., 2003). In this study, only the complete receptor binding domain of TcdA was shown to be functional, resulting in binding and endocytosis. Shortened receptor binding domain, although covering about 15 from 30 repetitive elements, showed no internalization. This finding does not argue against multiple receptor-binding subunits, but supports the view that at least the correct assembly and the correct folding of all CROPs are a prerequisite for receptor binding. Alternatively, it is conceivable that TcdA interacts with a single receptor molecule, meaning that despite the repetitive structure, only one unique cell receptor is recognized. The latter notion is supported by the finding that a monoclonal antibody that recognizes only two small epitopes in the 109 kDa receptor-binding domain completely blocks receptor binding and toxic effects (Lyerly *et al*., 1986; Frey and Wilkins, 1992).

### *Endocytosis and processing*

Subsequent to receptor binding, the toxins are endocytosed (Florin and Thelestam, 1983; Henriques *et al*., 1987) and the formed vesicles are acidified, which is a prerequisite for entering the cytosolic compartment (Figure 21.4). Inhibition of endosomal acidification by, e.g., bafilomycin A1 completely blocks cytotoxic effects, as shown for TcdA, TcdB, and TcsL. This inhibition can be bypassed by acidification of the culture medium (pH 5), allowing the translocation of the toxin directly across the cell membrane into the cytosol (Qa'Dan *et al*., 2000; Barth *et al*., 2001; Fiorentini and Thelestam, 1991).

At least TcdB is processed during the course of uptake (Pfeifer *et al*., 2003; Rupnik et al., 2005b). Only the enzymatically active fragment but not the holotoxin is detectable in the cytosol, and the translocation/binding domain remains in the endosomes (Pfeifer *et al*., 2003). The cleavage site $Leu_{543}/Gly_{544}$ was determined in $TcdB_{10463}$ after *in vitro* cleavage reaction (Rupnik *et al*., 2005b). The protease involved and the step where proteolytic cleavage takes place have not yet been identified. However, the *in vitro*

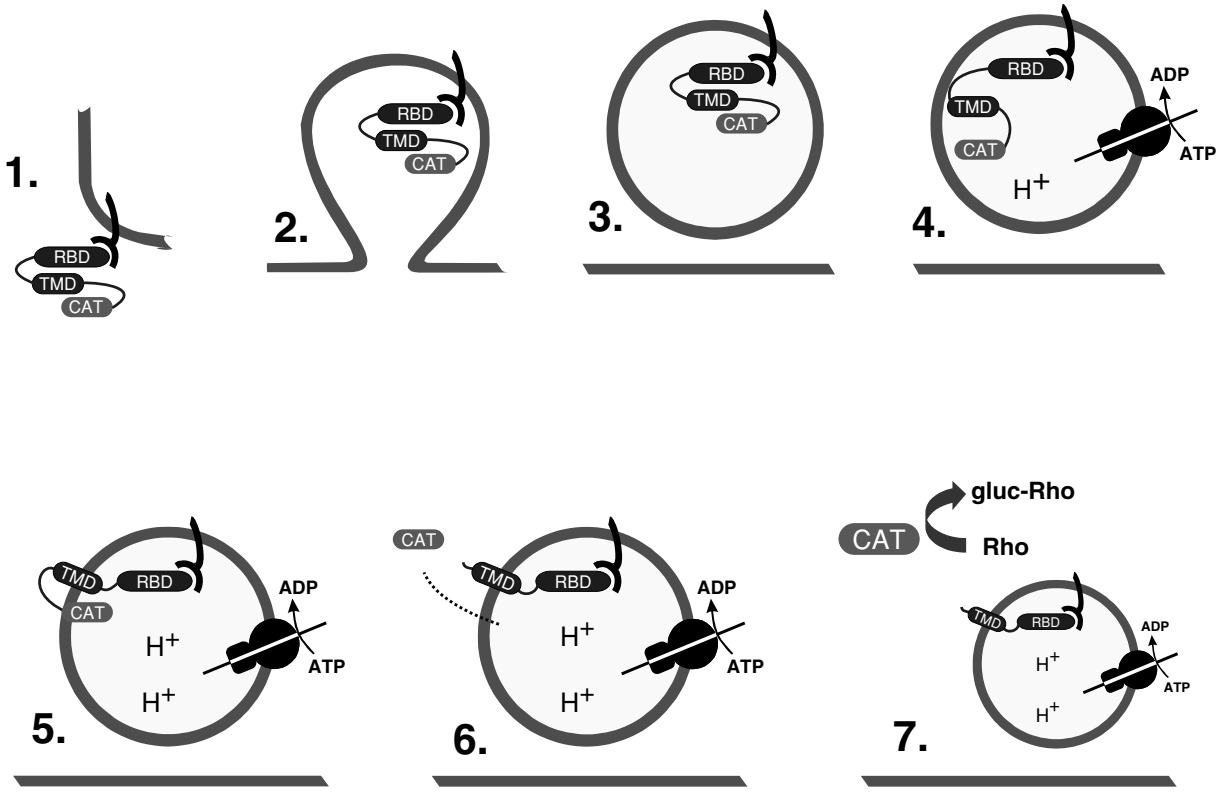



**FIGURE 21.4** Cell entry of LCTs exemplified for TcdB. 1. The C-terminal receptor-binding domain (RBD) of TcdB interacts with the putative cell receptor. 2. Toxin binding induces receptor-mediated endocytosis. 3. Endocytosed vesicle is processed to endosomes. 4. Vesicular-ATPase pumps protons into the endosomal lumen, resulting in a decreased pH (about 5.5). 5. Acidic pH induces refolding of the toxin, allowing the hydrophobic transmembrane domain (TMD) to form a pore into the endosomal membranes (shown only for TcdB.). 6. The catalytic domain (cat) is translocated through the pore into the cytoplasm. The proteolytically cleavage catalytic domain is released (shown only for TcdB). 7. The catalytic domain mono-glucosylates the Rho-GTPase targets in the cytoplasm.

proteolytic activity, which results in a specific cleavage of TcdB in two fragments, has been partially characterized (Rupnik *et al*., 2005b). Based on the comparable primary structure, it is likely that other LCTs are also processed. The same cleavage site as in $TcdB_{10463}$ was determined also for variant $TcdB_{8864}$, with the difference at position -2 (EGSL/GEDD vs. EGAL/GEDD). Cleavage site EGAL/GEDD is conserved also in TcsL and the toxin could be cleaved *in vitro*, but further characterization of cleavage reaction was not done. Cleavage site is changed in TcdA (GGSL/SEDN) and TcnA (GRTL/NYED).

All LCTs carry in the middle part a hydrophobic region (Figure 21.1), which is thought to be involved in translocation. This notion is based on the known uptake mechanism of other toxins using an endosomal pathway, where the acidic endosomal pH triggers conformational changes in the hydrophobic stretch, allowing membrane insertions and subsequent translocation of the catalytic domain into the cytosol. That this notion is also true for TcdB is shown by the finding that a decrease in the pH, in fact, increases hydrophobicity of TcdB, a conformational prerequisite for membrane insertion (Qa'Dan *et al*., 2000). A further support comes from the finding that TcdB and TcsL are able to form pH-dependent pores, allowing small cations such as $Rb^+$ to pass (Barth *et al*., 2001). This seems to be a general property of TcdB since it can be reproduced in an artificial lipid bilayer model. The function of the pore is assumed to transport the catalytic domain from the endosomal lumen into the cytosol.

This model of the cell entry of a single-chained toxin seems not to be true for *C. sordellii* TcsL because TcsL consists of a dissociable protease-resistant high-molecular complex (Voth *et al*., 2004). Dissociation of this complex is pH-dependently regulated; at pH 4, distinct proteins of 45–55 kDa appear, which are cytotoxic.

## Molecular mode of action

All LCTs are transferases that catalyze the transfer of a glucose (glcNAc in the case of TcnA) moiety to cellular

targets, the Rho- and Ras-GTPases. Toxins TcdA and TcdB mono-glucosylate Rho, Rac, Cdc42, RhoG, and TC10, all GTPases that are members of the Rho subfamily (Just *et al*., 1995a; Just *et al*., 1995b; Boquet and Lemichez, 2003; Just and Gerhard, 2004). TcdA was reported also to partially modifiy Rap (Chaves-Olarte *et al*., 1997). TcsL (various isoforms) and the variant TcdBs from *C. difficile* strains 1470 and 8864 predominantly modify Rac from the Rho subfamily and, in addition, Ras-GTPases, such as Ras, Rap, and Ral (Genth *et al*., 1996; Popoff *et al*., 1996; Just *et al*., 1996; Hofmann *et al*., 1996; Schmidt *et al*., 1998; Chaves-Olarte *et al*., 1999; Chaves-Olarte *et al*., 2003). The glucose moiety from the ubiquitiously present co-substrate UDP-glucose is covalently bound to a pivotal threonine residue residing in the effector region of the GTPases (also called *switch 1*). The acceptor amino acids are Thr-37 in RhoA and Thr-35 in Rac, Cdc42, and Ras. TcnA from *C. novyi* recruits UDP-N-acetyl-glucosamine (UDPglcNAc) and transfers the glcNAc to the same threonine residue in Rho, Rac, and Cdc42 as toxins TcdA/TcdB (Selzer *et al*., 1996) (Table 21.2).

### *The glucosyltransferase activity*

*Catalytic domain*

The catalytic activity resides in the N-terminal part of the toxins, convincingly demonstrated for TcdA, TcdB, TcsL, and TcnA (Faust *et al*., 1998; Hofmann *et al*., 1997; Wagenknecht-Wiesner *et al*., 1997; Hoffman *et al*., 1998; Busch *et al*., 2000b). The studies using recombinant domains have shown that N-terminal domain covering amino acid residues 1 to 546 exhibited full enzyme activity. This is the minimum size of the catalytic domain since further reduction of the size results in loss of activity (Hofmann *et al*., 1997; Wagenknecht-Wiesner *et al*., 1997). The exact size of catalytic domain in TcdB (543 aa) was now defined by the characterization of the cleavage site (Rupnik *et al*., 2005b). Furthermore, microinjection of the N-terminal domain caused the same morphological and cytoskeletal effects as do the holotoxins.

These data have been fully supported by the application of chimeric toxins using a heterologous delivery system: The N-terminal part of the lethal factor of anthrax is fused to the catalytic domain of TcdB (residues 1–556) and the cell entry is mediated by the protective antigen of anthrax toxin (Spyres *et al*., 2001). The same system is applied to deliver the catalytic domain of TcsL from *C. sordellii* to cells (Voth *et al*., 2004). The finding that a mutant Don cell line—deficient of the co-substrate UDP-glucose—is resistant to the cytotoxins and that resistance is reversed by microinjection of UDP-glucose further supports the notion of the glucosyltransferase-based cytotoxicity (Chaves-Olarte *et al*., 1996). In conclusion, all the data support the notion that the cytotoxic activity of the LCTs is mediated by their inherent enzyme activity.

Structure-function analysis of the active fragments of *C. difficile* TcdB, *C. sordellii* TcsL, and *C. novyi* TcnA has shown that the essential structural element for enzyme activity is the D-X-D motif, an amino acid sequence of aspartic acid-any amino acid-aspartic acid (Busch *et al*., 1998) (Figure 21.5). This D-X-D motif, which is conserved in all LCTs, is present in various pro- and eukaryotic glycosyltransferases. UDP-glucose binding within the catalytic cleft of several eukaryotic glycosyltransferases is based on divalent metal ion-dependent coordination, which structurally requires two adjacent aspartic acids, the D-X-D motif (Wiggins and Munro, 1998). None of the aspartic acids, however, is identified to directly act as a catalytic amino acid (Boix *et al*., 2002). From the 3D structures of rabbit N-acetylglucosaminyltransferase and bovine beta1.4-galactosyltransferase, it can be deduced that one aspartate directly interacts with $Mn^{2+}$ to further mediate UDP binding (Unligil and Rini, 2000; Unligil *et al*., 2000; Boeggeman and Quasb, 2002). Exchange of one of the aspartic acid residues results in a more than 1000-fold decrease in enzyme activity, which cannot be rescued by an excess of $Mn^{2+}$ (Busch *et al*., 1998). Furthermore, such mutant toxin loses its ability to bind to the co-substrate UDP-glucose, shown by failure to be labeled with azido-UDP-glucose. In addition to the D-X-D motif, tryptophan-102 is also involved in binding of the co-substrate UDP-glucose, and mutational exchange results in a dramatic decrease in transferase activity (Busch *et al*., 2000a). These findings are not only important for the understanding of the catalytic

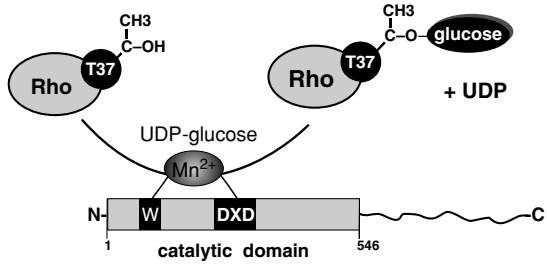



**FIGURE 21.5** Catalytic mechanism. The catalytic domain of the LCTs is located at the N-terminal part of the toxin molecule and recombinant functional proteins of 546 residues were used in the studies. The tryptophan-102 (W) and the D X D motif (residues 286–288) are involved in UDP-glucose co-substrate binding through $Mn^{2+}$ or $Mg^{2+}$. The C-terminal part (residues 408–468 in TcdB) covers the protein substrate recognition site. The catalytic domain forms together with the substrate Rho and the co-substrate UDP-glucose a ternary complex in which the glucose moiety is transferred to the acceptor threonine residue and covalently linked.

mechanism, but also serve as a basis for the generation of enzyme-deficient toxins.

*C. difficile* TcdA, TcdB, and the variant TcdBs, as well as TcsL and TcsH from *C. sordellii,* recruit the nucleotide sugar UDP-glucose as co-substrate; the glucose moiety is transferred to the protein substrate (Figure 21.5). *C. novyi* TcnA is an exception because it uses UDP-GlcNAc as a co-substrate. N-acetylglucosaminylation of small target GTPases, in fact, occurs in intact cells as it was deteced by specific [$^{14}$C]galactosylation of the cellular GTPase, which depends on the N-acetylglucosamine moiety attached (Selzer *et al*., 1996). Under artificial conditions, TcnA also utilizes UDP-glucose but the $K_m$ is about 340 μM compared to 17 μM for UDP-glucNAc and seems not to be of relevance (Busch *et al*., 2000b). In general, the $K_m$–value of the LCTs for nucleotide sugars is in the range 10–20 μM.

The catalytic mechanism implies a specific substrate recognition site for the Rho/Ras substrates. This site has been characterized by constructing chimeras of catalytic domains from toxins with different substrate specificities, such as TcdB and TcsL (Hofmann *et al*., 1998). Amino acid residues 408–468 in TcdB determine the specificity for Rho, Rac, and Cdc42, whereas residues 364–408 in TcsL determine for Rac and Cdc42 but not for Rho recognition. Interestingly, the substrate specificity of TcsL and TcdB is determined by different domains. The recognition of the Ras proteins in TcsL is mediated from the region aa 468–516, which is adjacent to the Rho recognition site (Hofmann *et al*., 1998; Busch and Aktories, 2000). It seems that the substrate recognition sites are modularly organized. However, the differing substrate specificities are in fact not understood. TcdA, TcdB, TcsH, and TcnA preferentially glucosylate Rho subfamily GTPases but not GTPases from other subfamilies (Table 21.2), whereas *C. sordellii* TcsL and the variant $TcdB_{1470}$ possess a different substrate specificity: They modify Rac but not Rho and, in addition, H-Ras, Rap, Ral, and R-Ras (Genth *et al*., 1996; Popoff *et al*., 1996; Just *et al*., 1996; Hofmann *et al*., 1996) (Table 21.2).

The acceptor residue threonine is highly conserved in Rho- and Ras-GTPase and plays an essential role in the GTP-driven switching of the effector region. Only in the GDP-bound form is the hydroxyl group accessible, and thus the inactive Rho-GDP is the preferred substrate for the cytotoxins (Just *et al*., 1995a; Herrmann *et al*., 1998).

*Catalytic mechanism*

The glucose is O-glycosidically bound in an α-anomerical configuration (Vetter *et al*., 2000; Geyer *et al*., 2003). Thus, the glucosylation reaction goes under retention of the configuration of the α-D-glucose at the C-1 position. Since 1.5-gluconolactone mimicking the transition state inhibits Ras glucosylation by TcsL, it can be concluded that the reaction mechanism includes a glucosyl oxonium transition state (Geyer *et al*., 2003). Retention of configuration and the glucosyl transition state exclude a single $S_N2$ reaction mechanism but rather argues for a binucleophilic substitution (double replacement) or a stereospecific $S_N1$ reaction, where structural constraints of the catalytic pocket prevent formation of a racemate (Vetter *et al*., 2000; Geyer *et al*., 2003).

In addition to the transferase activity, LCTs exhibit glycohydrolase activity in the absence of Rho substrates to hydrolytically cleave the co-substrate UDP-glucose into UDP and glucose (Just *et al*., 1995b; Chaves-Olarte *et al*., 1997; Ciesla, Jr. and Bobak, 1998; Busch *et al*., 2000a). The glycohydrolase activity is much slower than the transferase activity. Whether this hydrolase activity contributes to the biological activity of the LCTs is unclear. This activity, however, can be used for studying the requirement of the divalent cations for enzyme activity, because this a well-defined, two-component system merely exists of the enzyme (cytotoxin) and the nucleotide sugar but is not hampered by the $Mg^{2+}$ ion-requiring Rho GTPases. $Mg^{2+}$ or $Mn^{2+}$ ions are essential for enzyme activity but not for correct folding because the removal of bound divalent metal ions by treatment with EDTA or EGTA results in a complete inhibition of enzyme activity, which can be fully restored by the addition of the metal ions (Just *et al*., 1996; Ciesla, Jr. and Bobak, 1998). Divalent metal ions are necessary but not sufficient for glycohydrolase activity since in the absence of $K^+$ cations, no enzyme activity is detectable (Ciesla, Jr. and Bobak, 1998). The requirement for $Mn^{2+}/Mg^{2+}$ and co-activation by $K^+$ may reflect that the LCTs are optimally adapted to the metal ion condition of the intracellular milieu of the mammalian target cell, especially because all factors are removed during the uptake process, including acidification and translocation through endosomal membranes.

### *Functional consequences of glucosylation of Rho-GTPases*

*Cellular functions of the substrates of the toxins*

The major cellular targets of the LCTs, the Rho GTPases, also called *small Rho GTP-binding proteins*, are molecular switches in various intracellular signaling pathways (Bishop and Hall, 2000; Takai *et al*., 2001b; Etienne-Manneville and Hall, 2002). The function of the switch is regulated by an intrinsic GTPase cycle (Figure 21.6). The active GTP-bound state is reached by catalysis of GEFs (guanine nucleotide exchange factors). This conformation of the Rho-GTPase allows

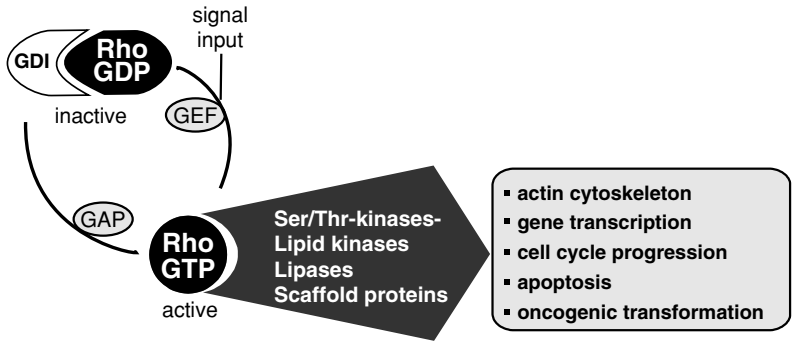



**FIGURE 21.6** Rho GTPase cycle. The small GTP-binding proteins are molecular switches, which are regulated by guanine nucleotide binding. The active GTP-bound state is reached by catalysis of GEFs (guanine nucleotide exchange factors), which are activated by external or internal signal input. The GTP-conformation allows binding to and activation of the effector proteins that amplify and execute the Rho signaling. Effector proteins are Ser/Thr-kinases, lipid kinases, lipases, and scaffold proteins. Inactivation is achieved by the intrinsic GTP-hydrolysing activity—triggered by GAPs (GTPase activating proteins) —resulting in an inactive GDP-bound Rho incapable of downstream signaling. Inactive Rho is cytosolically entrapped by GDI (guanine nucleotide dissociation inhibitor).

binding to and activation of the effector proteins, which amplify and execute the Rho signaling. Effector proteins are Ser/Thr-kinases, lipid kinases, lipases, and scaffold proteins (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Zohn *et al*., 1998; Bishop and Hall, 2000; Ridley, 2001; Takai *et al*., 2001; Wettschureck and Offermanns, 2002; Etienne-Manneville and Hall, 2002). Inactivation is achieved by the intrinsic GTP-hydrolysing activity—triggered by other regulatory proteins such as GAPs (GTPase activating proteins)—resulting in an inactive GDP-bound Rho incapable of downstream signaling. Inactive Rho is cytosolically entrapped by GDI (guanine nucleotide dissociation inhibitor). This GTP-GDP cycle is governed by signal input through cell membrane-receptors and cross talk, respectively, and is accompanied by an additional spatial cycling from the cytosol (inactive state) to the membranes (active state) and back again (Figure 21.7A).

The family of Rho GTPases comprises Rho (A, B, C, D, G), Rac (1, 1b, 2, 3), Cdc42, Wnt-1, Chp, G25K, Rnd (1, 2, 3), TTF/RhoH, Rif, and TC10 (Van Aelst and D'Souza-Schorey, 1997; Mackay and Hall, 1998; Ridley, 2000). Best studied are RhoA, Rac, and Cdc42. RhoA induces formation of actin stress fibers and focal adhesions (Etienne-Manneville and Hall, 2002), Rac leads to formation of lamellipodia/membrane ruffles (Ridley *et al*., 1992), and Cdc42 induces formation of microspikes/filopodia (Kozma *et al*., 1995). Thus, Rho GTPases are essential for actin cytoskeletal-driven cell functions, such as cell migration, control of morphogenesis, and cell polarity. However, they are also involved in the regulation of cytoskeletal-independent cell functions, including cell cycle control, activation of transcription, apoptosis, and transformation (Bishop and Hall, 2000; Takai *et al*., 2001; Etienne-Manneville and Hall, 2002).

*Functional consequences of glucosylation of Rho GTPases*

The glucose moiety attached to the conserved threonine residue in the effector region causes various alterations of Rho functions (Figure 21.7): (i) Rho activation by exchange factors (GEFs) is reduced; (ii) intrinsic GTPase activity is reduced, but GAP-stimulated GTPase is completely inhibited; (iii) coupling to the effector proteins is completely blocked (Sehr *et al*., 1998; Herrmann *et al*., 1998). Inhibition of effector coupling is based on the effect of the glucose moiety to stabilize the inactive conformation of the effector region, although GTP can be bound (Vetter *et al*., 2000). In addition to the GTPase cycling, also the spatial cycling of the Rho-GTPases from cytosol to membranes is altered (Genth *et al*., 1999). Surprisingly, glucosylation renders Rho properties so that GDP-bound glucosylated Rho is bound to membranes, but cannot be complexed to GDI any more. Glucosylation blocks the cytosol-membrane cycling of Rho proteins, thereby leading to an entrapment at the membranes, which is thought to be the basis of the dominant negative effect of glucosylated Rho (Genth *et al*., 1999). Thus, glucosylation redundantly switches off Rho signaling to completely block all Rho-dependent signal pathways. The entrapment at the membranes, however, indicates a gain of function as a negative regulator rather than a mere inactivation of the GTPases.

The cytotoxic activity of the toxins caused by actin depolymerization can be fully explained by the inactivation of Rho-GTPases. RhoA is the upstream activator of the serine/threonine kinase ROCK (Rho-kinase), which in cooperation with the scaffold protein mDia regulate actin-myosin assembly and contractility, as well as actin polymerization, resulting in alteration of stress fiber formation and cell motility. Rac1

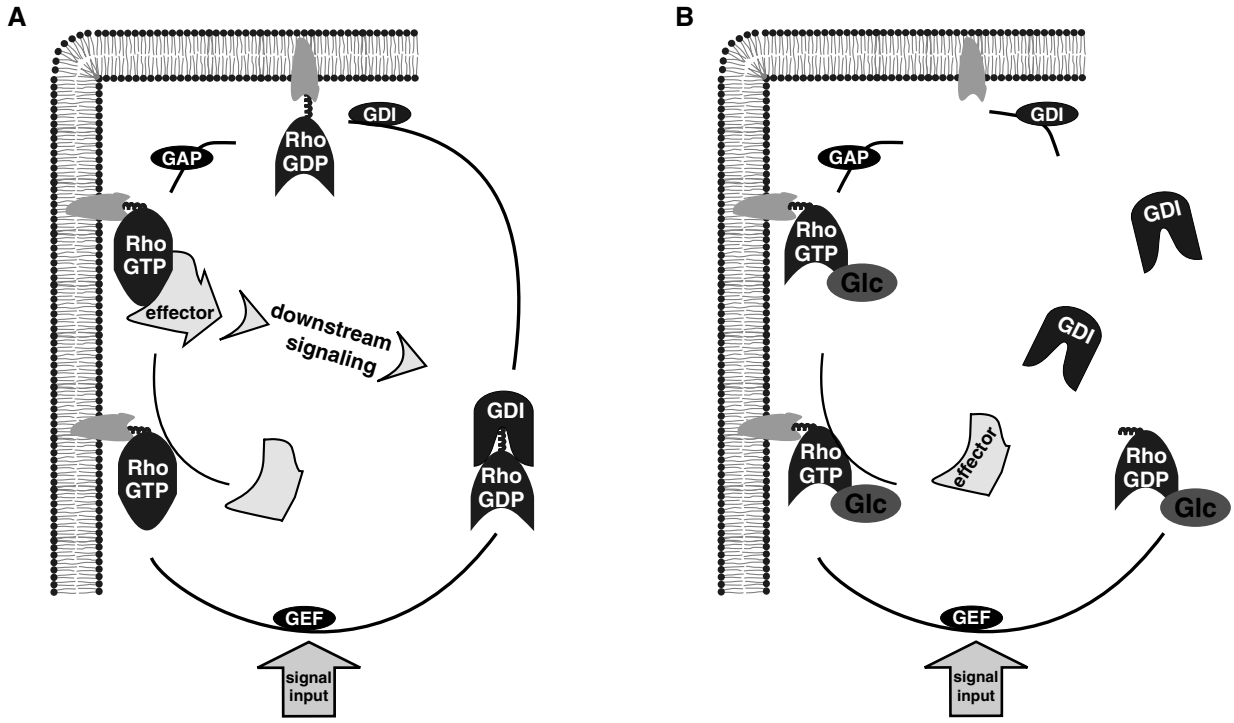



**FIGURE 21.7** Functional consequences of glucosylation. **A.** Cellular Rho signaling is governed by the GTP-GDP (GTPase)-cycle, which is driven by regulatory proteins such as GEFs, GAPs, and GDI. The GTPase cycle is accompanied by a spatial cycling from the cytosol (inactive state, complexed with GDI) to the membranes (active state) and back again. **B.** LCT-catalyzed mono-glucosylation alters the properties of Rho-GTPases. Glucosylated Rho is loaded with GTP and is translocated to membranous binding sites. Interaction with GAP and binding to GDI is blocked so that glucosylated Rho is entrapped at the membranes. However, glucosylated Rho is incapable of binding to effector proteins, resulting in a complete inhibition of Rho downstream signaling. Thus, the hallmark of glucosylated Rho is the enrichment of signaling incapable Rho at the membranes.

acts through PAK/WAVE/$PIP_2$, whereas Cdc42 acts through WASP. Their inactivation causes actin depolymerization in filopodia and lamellipodia, as well as de-inhibition of F-actin stabilization, resulting in inhibition of cell motility and phagocytosis. However, the molecular mode of action—glucosylation of Rho GTPases—implicates that all Rho-regulated cell functions are altered and, in fact, numerous cellular effects have been described to be based on inactivation of Rho GTPases: secretion (Prepens *et al*., 1996), phospholipase D activity (Schmidt *et al*., 1996), phagocytosis (Caron and Hall, 1998), neuronal axon formation (Bradke and Dotto, 1999), apoptosis (Subauste *et al*., 2000), deregulation of neurotransmitter exocytosis (Doussau *et al*., 2000), calcium mobilization (Djouder *et al*., 2000), muscarinic receptor signaling (Linseman *et al*., 2000), and chemoattractant receptor signaling (Servant *et al*., 2000).

Both TcdA and TcdB from the reference strain VPI 10463 cause morphological changes of fibroblasts, characterized by cell rounding and intermediate formation of "neurite-like" protrusions, but the intoxicated cells remain attached to the substratum. In contrast, variant TcdBs (e.g., $TcdB_{1470}$ and $TcdB_{8864}$) and TcsL from *C. sordellii* induce rounding with formation of some filopodia-like structures, however, accompanied by clustering of the cells followed by detachment (Chaves-Olarte *et al*., 2003) (Figure 21.8). The TcsL and the related variant TcdBs glucosylate Rac1 and Ras-GTPases but not RhoA. R-Ras, but not Rho, is involved in the control of cell adhesiveness through modulation of the integrin avidity (Zhang *et al*., 1996) and, thus, the difference in substrate specificity is responsible for different cytotoxic outcomes (Chaves-Olarte *et al*., 2003).

### *Enzyme-independent properties of* C. difficile *TcdA and TcdB*

Mitochondrial targeting of TcdA is currently under debate as an enzyme-independent activity important for the inflammatory activity of the cytotoxins (He *et al*., 2002). The release of interleukin-8 (IL-8) from colonocytes was reported to be related to the damage of mitochondria caused by TcdA. Toxin-induced damage resulted in a decreased ATP production and

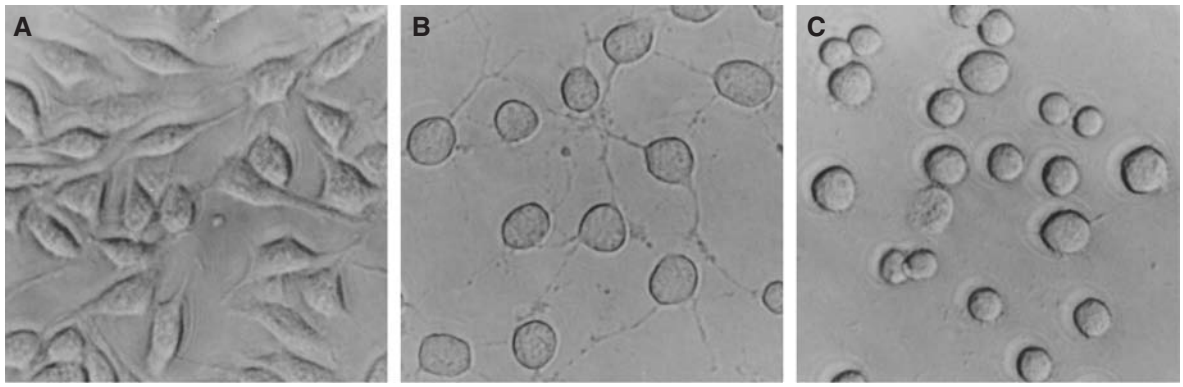



**FIGURE 21.8** Two morphological types of cytopathic effects (CPE) caused by LCTs. **(A)** Untreated control McCoy cells. **(B)** Difficile-like CPE with cells showing long protrusions; this CPE is obtained by toxins produced by the reference *C. difficile* strain and most of the variant *C. difficile* toxins (toxinotypes I–VII, XII–XIII, XV–XVI, XVIII–XXIV). **(C)** Sordellii-like CPE caused by TcdL and variant TcdBs of $A^-B^+$ toxinotypes (VIII, X, and XVII) and $A^+B^+$ toxinotypes (IX and XIV).

formation of reactive oxygene species (ROS), inducing a sequence of reactions involving activation of NFκB and eventually releasing the proinflammatory cytokine IL-8. Since this sequence takes place in a time window in which no glucosylation of Rho-GTPases were detectable, a Rho-independent effect of TcdA was postulated. This notion was supported by the finding that isolated mitochondria were directly affected by TcdA (He *et al*., 2000; He *et al*., 2002). However, the contrary finding has been reported, showing that mitochondrial damage as initiation of apoptosis started after 18–24 h of intoxication and was clearly dependent on the enzyme actitivity of the toxin (Brito *et al*., 2002). The effect of TcsL on mitochondria independently of the enzymatic effect on small GTPases was also reported (Petit *et al*., 2003)

A further Rho-independent effect is the rapid activation of the MAP-kinases ERK, p38-kinase, and JNK, starting 1–2 min after TcdA application to the monocytic THP-1 cell line (Warny *et al*., 2000). This early activation of kinase cascades clearly occurs before endocytosis of TcdA and the intracellular glucosylation of the Rho-GTPases, and it may be triggered through an interaction of the toxin with the host cell receptor. The p38-kinase activation resulted in monocyte activation and IL-8 production, thus offering an explanation of how TcdA may cause inflammatory processes.

The holotoxin TcdB is able to induce apoptosis in a caspase-dependent and caspase-independent manner. However, when the catalytic domain of TcdB—delivered as chimeric anthrax toxin or expressed in cells—was applied instead of the holotoxin, apoptosis was exclusively induced via a caspase-independent pathway (Qa'Dan *et al*., 2002). Thus, different TcdB domains should trigger apoptosis by two different mechanisms.

TcdA is able to induce a strong and long-lasting upregulation of the immediate early gene product RhoB at least partially through the p38-MAP-kinase signal pathway. Newly expressed RhoB is only partially inactivated by glucosylation, so that a significant portion of RhoB is active and capable of downstream signaling (Gerhard *et al*., 2005). Thus, the paradigm has to be challenged that the LCTs are specific inhibitors of the small GTPases.

In conclusion, it appears that especially TcdA and TcdB possess additional enzyme-independent biological activities that may contribute to pro-inflammatory effects in the course of the pseudomembranous colitis.

## LARGE CLOSTRIDIAL CYTOTOXINS IN PATHOGENESIS

Although all large clostridial cytotoxins possess glucosyltransferase activity to inactivate Rho- and Ras-GTPases, their *in vivo* effects differ from each other. They are major virulence factors causative for different diseases and clinical outcomes, respectively. TcdA and TcdB from *C. difficile* cause the antibiotic-associated diarrhea and the severe form, the pseudomembranous colitis (Kelly *et al*., 1994; Bartlett, 1994; Kelly and LaMont, 1998) is TcsL from *C. sordellii* involved in diarrhea and enterotoxemia in domestic animals and in gas gangrene in man (Hatheway, 1990), *C. novyi* TcnA has been identified as a causative agent for gas gangrene infections in man and animals (Hatheway, 1990). The difference between the comparable mode of action but different clinical features is likely based on the different organ targeting of the toxin-producing bacteria that colonize the gut or injured organs.

Additional strain-specific virulence factors are most likely involved in the pathogenesis.

Based on early animal studies, *C. difficile* TcdA, a biologically active enterotoxin, is thought to act primarily, whereas the effects of TcdB are secondary to TcdA (Lyerly *et al*., 1985). However, in human disease it appears that both toxins are of comparable relevance (Savidge *et al*., 2003). In about 5–7% of cases of *C. difficile*-associated diarrhea, so-called variant $A^-B^+$ strains are involved; these strains produce a variant TcdB, but no functional TcdA (von Eichel *et al.*, 1999; Kuijper *et al*., 2001; Barbut *et al*., 2002; Rupnik *et al*., 2003; Johnson *et al*., 2003). Despite lack of production of putative primary acting TcdA, the $A^-B^+$ strains are clearly associated with mild and severe forms of disease in humans. However, such strains are not able to induce diarrhea and PMC in animals (Depitre *et al*., 1993, Sambol *et al*., 2001), which further questions the relevance of the animal model to study the human disease.

The clinical symptoms of pseudomembranous colitis are characterized by secretory diarrhea and inflammatory processes of colonic mucosa. Toxin-induced fluid response and diarrhea may be explained by a decrease in barrier function of the colonocytes based on disruption of the actin cytoskeleton and inhibition of the function of tight junctions, resulting in a decreased transepithelial resistance allowing paracellular fluid loss (Hecht *et al*., 1988; Moore *et al*., 1990; Hecht *et al*., 1992; Riegler *et al*., 1995; Nusrat *et al*., 1995; Gerhard *et al*., 1998; Feltis *et al*., 2000; Nusrat *et al*., 2001; Liu *et al*., 2003).

The inflammatory response to the toxins is characterized by massive neutrophil infiltration and the production and release of various inflammatory mediators from mast cells and macrophages, such as prostaglandins and leukotriens, as well as IL-1, IL-6, IL-8, TNF-α, and IFN-γ (Pothoulakis *et al*., 1988; Mahida *et al*., 1996; Jefferson *et al*., 1999; Warny *et al*., 2000; He *et al*., 2002; Savidge *et al*., 2003; Ishida *et al*., 2004; Pothoulakis *et al*., 1998; Thelestam and Chaves-Olarte, 2000; Pothoulakis and LaMont, 2001). Furthermore, the enteric nervous system (ENS) is activated by the toxins through an unknown sequence of steps triggering secretory diarrhea and amplifying inflammation (Pothoulakis *et al*., 1998; Farrell and LaMont, 2000; Mantyh *et al*., 2000; Jones and Blikslager, 2002; Neunlist *et al*., 2003). However, the cellular and molecular mechanisms through which the toxins induce inflammatory processes are less clear and cannot be satisfactorily explained by their intracellular enzyme activity to inactivate small GTPases (Farrell and LaMont, 2000; Poxton *et al*., 2001; Bartlett, 2002; Stoddart and Wilcox, 2002).

## APPLICATION OF THE CYTOTOXINS AS TOOLS IN CELL BIOLOGY

Some of the large clostridial cytotoxins are applied as tools in cell biological research to study Rho-dependent signal cascades in intact cells. The basis for application is the cell accessibility (self-managed cell entry by receptor-mediated endocytosis) of the toxins and the notion that the cytotoxins possess a confined substrate specificity allowing the identification of the GTPase isoform involved in the cell function studied. Cell lines exhibit a broad spectrum of sensitivity towards the large clostridial cytotoxins. For example, CHO cells are highly sensitive to TcdB acting at femto-molar range, whereas HEp2 cells are quite insensitive; most fibroblasts and epithelial cells are insensitive to TcsL, but RBL (mast) cells are highly sensitive. TcdB is usually used, as it is much more potent than TcdA. To check whether Rac but not RhoA is involved or to check the participation of Ras-like GTPases, TcsL or the variant $TcdB_{1470}$ is applied because of their different target spectrum (Table 21.2). Although the canonical substrates of TcdA and TcdB are thought to be only Rho, Rac, and Cdc42, more Rho-GTPase isoforms are glucosylated than generally appreciated (Table 21.2). Especially RhoG and TC10 have to be considered.

The morphological changes (cell rounding and formation of arborized morphology) induced by the LCTs are an excellent readout to check cell sensitivity and to check whether the toxin applied has done its job in the special experimental setting. Non-adherent cells, however, escape the readout system of cell-shape changes. In this case, the only reliable method to check the effectiveness of the toxin is to perform a differential glucosylation. The principle of this assay is the fact that toxin-catalyzed glucosylation in intact cells prevents a second [$^{14}$C]glucosylation (co-substrate UDP-[$^{14}$C]glucose) of the lysates. A decrease in radioactive labeling of the Rho-GTPases in lysates compared to non-treated cells indicates previous glucosylation in the intact cell and thus proves the effect of the toxin. An alternative is the application of the C3-catalyzed [$^{32}$P]ADP-ribosylation using [$^{32}$P]$NAD^+$ as co-substrate, which, however, only proves modification of Rho but not of Rac or Cdc42. The differential glucosylation and ADP-ribosylation, respectively, are also applicable to assess the extent of inactivated (glucosylated) Rho-GTPases. However, one has to consider that membranous Rho is the active one, so that assessing glucosylation of membranous Rho is more relevant.

Since the Rho-GTPases are the master regulators of the actin cytoskeleton, the cytotoxins alter every actin-based cell function. To distinguish between mere

cytoskeletal and non-cytoskeletal effects, agents or toxins that directly interfere with actin polymerization should be applied for control. One such agent is latrunculin B, which enters cells by diffusion and sequesters G-actin. The second is the binary C2 toxin from *C. botulinum*, which ADP-ribosylates G-actin, thereby causing permanent depolmerization of the actin filaments (Aktories *et al*., 1997b; Barbieri *et al*., 2002; Aktories *et al*., 1997a).

Thus, the large clostridial cytotoxins should only be used for the initial orienting studies. The findings have to be confirmed by other methods, such as expression of dominant negative GTPases or application of siRNA, (Just *et al*., 1997; Bobak, 1999; Just and Boquet, 2000; Schiavo and van der Goot, 2001; Boquet, 2002).

## ACKNOWLEDGMENTS

Authors are supported by the Deutsche Forschungsgemeinschaft (SFB 621) (IJ), Slovenian Research Agency Project J1-6456 (MR), and Alexander von Humboldt Foundation (MR).

## REFERENCES

Ackermann, G., Loffler, B., Tang-Feldman, Y.J., Cohen, S.H. and Rodloff, A.C. (2004). Cloning and expression of Clostridium difficile toxin A gene (tcdA) by PCR amplification and use of an expression vector. *Mol. Cell. Probes* **18,** 271–274.

Aktories, K., Prepens, U., Sehr, P. and Just, I. (1997a). Probing the actin cytoskeleton by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin. In: *Bacterial Toxins* (ed. K.Aktories) pp. 129–139. Chapman & Hall, Weinheim.

Aktories, K., Sehr, P. and Just, I. (1997b). Actin-ADP-ribosylating toxins: cytotoxic mechanism of *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin. In: *Bacterial Toxins* (ed. K.Aktories) pp. 93–101. Chapman & Hall, Weinheim.

Ball, D.W., van Tassell R.L., Denton Roberts M., Hahn P.E., Lyerly, D.M. and Wilkins T.D. (1993) Purification and characterization of alpha-toxin produced by *Clostridium novyi* type A. *Infect.Immun*. **61,** 2912–2918.

Barbieri, J.T., Riese, M.J. and Aktories, K. (2002). Bacterial toxins that modify the actin cytoskeleton. *Annu. Rev. Cell Dev. Biol*. **18,** 315–344.

Barbut, F., Lalande, V., Burghoffer, B., Thien, H.V., Grimpel, E. and Petit, J.C. (2002). Prevalence and genetic characterization of toxin A variant strains of Clostridium difficile among adults and children with diarrhea in France. *J. Clin. Microbiol*. **40,** 2079–2083.

Barc, M.C., Depitre, C., Corthier, G., Collignon, A., Su, W.J., Bourlioux, P. (1992) Effects of antibiotics and other drugs on toxin production in *Clostridium difficile in vitro* and *in vivo. Antimic. Agent. Chemother*. **36,** 1332–1335.

Barroso, L.A., Moncrief, S.J., Lyerly, D.M. and Wilkins, T.D. (1994). Mutageneis of the *Clostridium difficile* toxin B gene and effect on cytotoxic activity. *Microb. Pathog*. **16,** 297–303.

Barth, H., Pfeifer, G., Hofmann, F., Maier, E., Benz, R. and Aktories, K. (2001). Low pH-induced formation of ion channels by *Clostridium difficile* toxin B in target cells. *J. Biol. Chem*. **276,** 10670–10676.

Bartlett, J.G. (1994). *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. *Clin. Infect. Dis*. **18,** 265–272.

Bartlett, J.G. (2002). Antibiotic-associated diarrhea. *New England J. Med*. **346,** 334–339.

Bette, P., Oksche A., Mauler F., von Eichel-Streiber C., Popoff M. and Habermann E. (1991). A comparative biochemical, pharmacological, and immunological study of *Clostridium novyi* α-toxin, *C.difficile* toxin B, and *C.sordellii* lethal toxin. *Toxicon* **29,** 877–887.

Bishop, A.L. and Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem. J.* **348,** 241–255.

Blake, J.E., Mitsikosta, F. and Metcalfe, M.A. (2004). Immunological detection and cytotoxic properties of toxins from toxin A-positive, toxin B-positive *Clostridium difficile* variants. *J. Med. Microbiol*. **53,** 197–205.

Bobak, D.A. (1999). Clostridial toxins: Molecular probes of Rho-dependent signaling and apoptosis. *Mol. Cell Biochem*. **193,** 37–42.

Boeggeman, E. and Quasb, P.K. (2002). Studies on the metal binding sites in the catalytic domain of beta1, 4-galactosyltransferase. *Glycobiology* **12,** 395–407.

Boix, E., Swaminathan, J., Zhang, Y., Natesh, R., Brew, K. and Acharya, K.R. (2002). Structure of UDP complex of UDP-galactose:a-galactoside-a-1, 3-galactosyltransferase at 1.53-A resolution reveals a conformational change in the catalytic important C terminus. *J. Biol. Chem*. **276,** 48608–48614.

Boquet, P. (2002). Modification of small GTP-binding proteins by bacterial protein toxins. *Methods Microbiol*. **31,** 225–244.

Boquet, P. and Lemichez, E. (2003). Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis ? *Trends Cell Biol*. **13,** 238–246.

Boriello, S.P., Wren, B.W., Hyde, S., Seddon, S.V., Sibbons, P., Krishna, M.M., Tabaqchali, S., Manek, S. and Price, A.B. (1992). Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect. Immun*. **60,** 4192–4199.

Bradke, F. and Dotto, G.P. (1999) The role of local actin instability in axon formation. *Science* **283,** 1931–1934.

Braun, V., Hundsberger, T., Leukel, P., Sauerborn, M. and Eichel-Streiber, C.v. (1996). Definition of the single integration site of the pathogenicity locus in *Clostridium difficile. Gene* **181,** 29–38.

Braun, V., Mehlig, M., Moos, M., Rupnik, M., Kalt, B., Mahony, D.E. and Eichel-Streiber, C.V. (2000). A chimeric ribozyme in *Clostridium difficile* combines features of group I introns and insertion elements. *Mol. Microbiol*. **36,** 1447–1459.

Brito, G.A.C., Fujji, J., Carneiro, B.A., Lima, A.A.M., Obrig, T. and Guerrant, R.L. (2002). Mechanism of Clostridum difficile toxin A-induced apoptosis in T84 cells. *J. Infect. Dis*. **186,** 1438–1447.

Burland, V., Shao, Y., Perna, N.T., Plunkett, G., Sofia, H.J. and Blattner, F.R. (1998). The complete DNA sequence analysis of the large virulence plasmid of *Escherichia coli* O157:H7. *Nucl. Acid Res*. **26,** 4196–4204.

Burger, S., Tatge, H., Hofmann, F., Just, I. and Gerhard, R. (2003). Expression of recombinant Clostridium difficile toxin A using the Bacillus megaterium system. *Biochem. Biophys. Res. Commun*. **307,** 584–588.

Busch, C. and Aktories, K. (2000). Microbial toxins and the glycosylation of rho family GTPases. *Curr. Opin. Struct. Biol*. **10,** 528–535.

Busch, C., Hofmann, F., Gerhard, R. and Aktories, K. (2000a). Involvement of a conserved tryptophan residue in the UDP-glucose binding of large clostridial cytotoxin glycosyltransferases. *J. Biol. Chem*. **275,** 13228–13234.

Busch, C., Hofmann, F., Selzer, J., Munro, J., Jeckel, D. and Aktories, K. (1998). A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial cytotoxins. *J. Biol. Chem*. **273,** 19566–19572.

Busch, C., Schömig, K., Hofmann, F. and Aktories, K. (2000b). Characterization of the catalytic domain of *Clostridium novyi* alpha-toxin. *Infect. Immun.* **68,** 6378–6383.

Caron, E. and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282,** 1717–1721.

Chaves-Olarte, E., Weidmann, M., Von Eichel-Streiber, C. and Thelestam, M. (1997). Toxins A and B from *Clostridium difficile* differ with respect to enzymatic potencies, cellular substrate specificities, and surface binding to cultured cells. *J. Clin. Invest.* **100,** 1734–1741.

Chaves-Olarte, E., Löw, P., Freer, E., Norlin, T., Weidmann, M., Von Eichel-Streiber, C. and Thelestam, M. (1999). A novel cytotoxin from *Clostridium difficile* serogroup F is a functional hybrid between two other large clostridial cytotoxins. *J. Biol. Chem.* **274,** 11046–11052.

Chaves-Olarte, E., Freer, E., Parra, A., Guzmán-Verri, C. Moreno, E. and Thelestam, M. (2003). R-Ras glucosylation and transient RhoA activation determine the cytopathic effect produced by toxin B variants from toxin A-negative strains of Clostridium difficile. *J. Biol. Chem.* **278,** 7956–7963.

Ciesla, W.P., Jr. and Bobak, D.A. (1998). Clostridium difficile toxins A and B are cation-dependent UDP-glucose hydrolases with differing catalytic activities. *J. Biol. Chem.* **273,** 16021–16026.

Depitre, C., Delmee, M., Avesani, V., L'Haridon, R., Roels, A., Popoff, M. and Corthier, G. (1993). Serogroup F strains of *Clostridium difficile*produce toxin B but not toxin A. *J. Med. Microbiol.* **38,** 434–441.

Djouder, N., Prepens, U., Aktories, K. and Cavalié, A. (2000). Inhibition of calcium release-activated calcium current by Rac/Cdc42-inactivating clostridial cytotoxins in RBL cells. *J. Biol. Chem.* **275,** 18732–18738.

Doussau, F., Gasman, S., Humeau, Y., Vitiello, F., Popoff, M., Boquet, P., Bader, M.-F. and Poulain, B. (2000). A Rho-related GTPase is involved in $Ca^{2+}$-dependent neurotransmitter exocytosis. *J. Biol. Chem.* **275,** 7764–7779.

Dove, C.H., Wang, S.-Z., Price, S.B., Phelps, C.J., Lyerly, D.M., Wilkins, T.D. and Johnson, J.L. (1990). Molecular characterization of the *Clostridium difficile* toxin A gene. *Infect. Immun.* **58,** 480–488.

Drummond, L.J., Smith, D.G.E. and Poxton, I.R. (2003). Effects of sub-MIC concentrations of antibiotics on growth of and toxin production by *Clostridium difficile*. *J. Med. Microbiol.* **52,** 1033–1038.

Dupuy, B. and Sonenshein, L. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Mol. Microbiol.* **27,** 107–120.

Dupuy, B., Mani, N., Katayama, S. and Sonenshein, A.L. (2005). Transcription activation of a UV-inducible Clostridium perfringens bacteriocin gene by a novel σ factor. *Mol. Microbiol.* **55,** 1196–1206.

Eklund, M.W. (1993). The role of bacteriophages and plasmids in the production of toxins and other biologically active substances by Clostridium botulinum and Clostridium novyi. In: *Genetics and Molecular Biology of the Anaerobic Bacteria*. (ed. M. Sebald) pp. 179–194. Springer-Verlag, New York.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420,** 629–635.

Farrell, R.J. and LaMont, J.T. (2000). Pathogenesis and clinical manifestations of Clostridium difficile diarhea and colitis. *Curr. Top. Microbiol. Immunol.* **250,** 109–125.

Faust, C., Ye, B. and Song, K.-P. (1998). The enzymatic domain of *Clostridium difficile* toxin A is located within its N-terminal region. *Biochem. Biophys. Res. Commun.* **251,** 100–105.

Feltis, B.A., Wiesner, S.M., Kim, A.S., Erlandsen, S.L., Lyerly, D.L., Wilkins, T.D. and Wells, C.L. (2000). Clostridium difficile toxins A and B can alter epithelial permeability and promote bacterial paracellular migration through HT-29 enterocytes. *Shock* **14,** 629–634.

Fiorentini, C. and Thelestam, M. (1991). Clostridium difficile toxin A and its effects on cells. *Toxicon* **29,** 543–567.

Florin, I. and Thelestam, M. (1983). Internalization of *Clostridium difficile* cytotoxin into cultured human lung fibroblasts. *Biochim. Biophys. Acta* **763,** 383–392.

Frey, S.M. and Wilkins, T.D. (1992). Localization of two epitopes recognized by monoclonal antibody PCG-4 on *Clostridium difficile* toxin A. *Infect. Immun.* **60,** 2488–2492.

Frisch, C., Gerhard, R., Aktories, K., Hofmann, F. and Just, I. (2003). The complete receptor-binding domain of Clostridium difficile toxin A is required for endocytosis. *Biochem. Biophys. Res. Commun.* **300,** 706–711.

Genth, H., Hofmann, F., Selzer, J., Rex, G., Aktories, K. and Just, I. (1996). Difference in protein substrate specificity between hemorrhagic toxin and lethal toxin from *Clostridium sordellii*. *Biochem. Biophys. Res. Commun.* **229,** 370–374.

Genth, H., Aktories, K. and Just, I. (1999). Monoglucosylation of RhoA at Threonine-37 blocks cytosol-membrane cycling. *J. Biol. Chem.* **274,** 29050–29056.

Gerhard, R., Schmidt, G., Hofmann, F. and Aktories, K. (1998). Activation of Rho GTPases by Escherichia coli cytotoxic necrotizing factor 1 increases intestinal permeability in Caco-2 cells. *Infect. Immun.* **66,** 5125–5131.

Gerhard, R., Tatge, H., Genth, H., Thum, T., Borlak, J., Fritz, G. and Just, I. (2005). Clostridium difficile toxin A induces expression of the stress-induced early gene product RhoB. *J. Biol. Chem.* **280,** 1499–1505.

Geyer, M., Wilde, C., Selzer, J., Aktories, K. and Kalbitzer, H.R. (2003). Glucosylation of Ras by Clostridium sordellii lethal toxin: consequences for effector loop conformations observed by NMR spectroscopy. *Biochemistry* **42,** 11951–11959.

Green, G.A., Schué, V. and Monteil, H. (1995). Cloning and characterization of the cytotoxin L-encoding gene of *Clostridium sordellii*: homology with *Clostridium difficile* cytotoxin B. *Gene* **161,** 57–61.

Green, G.A., Schue, V., Girardot, R. and Monteil, H. (1996). Characterization of an enterotoxin-negative, cytotoxin-positive strain of *Clostridium difficile*. *J. Med. Microbiol.* **44,** 60–64.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279,** 509–514.

Hammond, G.A. and Johnson, J.L. (1995). The toxinogenic element of *Clostridium difficile* strain VPI 10463. *Microbial. Pathog.* **19,** 203–213.

Hammond, G.A., Lyerly, D.M. and Johnson, J.L. (1997). Transcriptional analysis of the toxigenic element of *Clostridium difficile*. Microb. Pathogen. **22,** 143–154.

Haslam, S.C., Ketley, J.M., Mitchell, T.J., Stephen, J., Burdon, D.W. and Candy, D.C.A. (1986). Growth of *Clostridium difficile* and production of toxins A and B in complex and defined medium. *J. Med. Microbiol.* **21,** 293–297.

Hatheway, C.L. (1990). Toxigenic clostridia. *Clin. Microbiol. Rev.* **3,** 66–98.

He, D., Hagen, J., Pothoulakis, C., Chen, M., Medina, N.D., Warny, M. and LaMont, J.T. (2000). Clostridium difficile toxin A causes early damage to mitochondria in cultured cells. *Gastroenterology* **119,** 139–150.

He, D., Sougioultzis, S., Hagen, S., Liu, J., Keates, S., Keates, A.C., Pothoulakis, C. and LaMont, J.T. (2002). Clostridium difficile toxin triggers human colonocyte IL-8 release via mitochondrial oxygen radical generation. *Gastroenterology* **122,** 1048–1057.

Hecht, G., Pothoulakis, C., LaMont, J.T. and Madara, J.L. (1988). *Clostridium difficile* toxin A pertubs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. *J. Clin. Invest.* **82,** 1516–1524.

Hecht, G., Koutsouris, A., Pothoulakis, C., LaMont, J.T. and Madara, J.L. (1992). *Clostridium difficile* toxin B disrupts the barrier function of $T_{84}$ monolayers. *Gastroenterology* **102,** 416–423.

Henriques, B., Florin, I. and Thelestam, M. (1987). Cellular internalization of *Clostridium difficile* toxin A. *Microb. Pathogen*. **2,** 455–463.

Herrmann, C., Ahmadian, M.R., Hofmann, F. and Just, I. (1998). Functional consequences of monoglucosylation of H-Ras at effector domain amino acid threonine-35. *J. Biol. Chem*. **273,** 16134–16139.

Hofmann, F., Herrmann, A., Habermann, E. and Eichel Streiber, C.v. (1995). Sequencing and analysis of the gene encoding the α-toxin of *Clostridium novyi* proves its homology to toxins A and B of *Clostridium difficile*. *Mol. Gen. Genet*. **247,** 670–679.

Hofmann, F., Rex, G., Aktories, K. and Just, I. (1996). The Ras-related protein Ral is monoglucosylated by *Clostridium sordellii* lethal toxin. *Biochem. Biophys. Res. Commun*. **227,** 77–81.

Hofmann, F., Busch, C., Prepens, U., Just, I. and Aktories, K. (1997). Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. *J. Biol. Chem*. **272,** 11074–11078.

Hofmann, F., Busch, C. and Aktories, K. (1998). Chimeric clostridial cytotoxins: identification of the N-terminal region involved in protein substrate recognition. *Infect. Immun*. **66,** 1076–1081.

Hundsberger, T., Braun, V., Weidman, M., Leukel, P., Sauerborn, M. and Eichel-Streiber, C. V. (1997). Transcription analysis of the genes *tcdA–E* of the pathogenicity locus of *Clostridium difficile*. *Eur. J. Biochem*. **244,** 735–742.

Ishida, Y., Maegawa, T., Kondo, T., Kimura, A., Iwakura, Y., Nakamura, S. and Mukaida, N. (2004). Essential involvement of IFN-gamma in Clostridium difficile toxin A-induced enteritis. *J. Immunol*. **172,** 3018–3025.

Jefferson, K.K., Smith, M.F.Jr. and Bobak, D.A. (1999). Roles of intracellular calcium and NF-κB in the *Clostridium difficile* toxin A-induced up-regulation and secretion of IL-8 from human monocytes. *J. Immunol*. **163,** 5183–5191.

Johnson, J.L., Phelps, C., Barroso, L., Roberts, M.D., Lyerly, D.M. and Wilkins, T.D. (1990). Cloning and expression of the toxin B gene of *Clostridium difficile*. *Curr. Microbiol*. **20,** 397–401.

Johnson, S., Sambol, S.P., Brazier, J.S., Delmee, M., Avesani, V. and Gerding, D.N. (2003). International typing study of toxin A-negative, toxin B-positive Clostridium difficile variants. *J. Clin. Microbiol*. **41,** 1543–1547.

Jones, S.L. and Blikslager, A.T. (2002). Pole of the enteric nervous system in the pathophysiology of secretory diarrhea. *J. Vet. Intern. Med*. **16,** 222–228.

Just, I. and Boquet, P. (2000). Large clostridial cytotoxins as tools in cell biology. *Curr. Top. Microbiol. Immunol*. **250,** 97–107.

Just, I. and Gerhard, R. (2004). Large clostridial cytotoxins. *Rev. Physiol Biochem. Pharmacol*. **152,** 23–47.

Just, I., Hofmann, F. and Aktories, K. (2000). Molecular mode of action of the large clostridial cytotoxins. *Curr. Top. Microbiol. Immunol*. **250,** 55–83.

Just, I., Selzer, J., Hofmann, F. and Aktories, K. (1997). Clostridium difficile toxin B as a probe for Rho GTPases. *In: Bacterial Toxins—Tools in Cell Biology and Pharmacology* (ed. K. Aktories) pp. 159–168. Chapman & Hall, Weinheim.

Just, I., Selzer, J., Hofmann, F., Green, G.A. and Aktories, K. (1996). Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. *J. Biol. Chem*. **271,** 10149–10153.

Just, I., Selzer, J., Wilm, M., Von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995a). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375,** 500–503.

Just, I., Wilm, M., Selzer, J., Rex, G., Von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995b). The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J. Biol. Chem*. **270,** 13932–13936.

Karlsson, K.A. (1995). Microbial recognition of target-cell glycoconjugates. *Curr. Opin. Struct. Biol*. **5,** 622–635.

Kamiya, S., Ogura, H., Men, X.Q. and Nakamura, S. (1992). Correlation between cytotoxin production and sporulation in *Clostridium difficile*. *J. Med. Microbiol*. **37,** 206–210.

Karlsson S., Dupuy, B., Mukherjee, K., Norin, E., Burman, L.G. and Akerlund, T. (2003). Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. *Infect. Immun*. **71,** 1784–1793.

Karlsson, S., Burman, L.G. and Akerlund, T. (1999). Suppression of toxin production in *Clostridium difficile*VPI 10463 by amino acids. *Microbiology* **145,** 1683–1693.

Karlsson, S., Lindberg, A., Norin, E., Burman, L.G. and Akerlund, T. (2000). Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. *Infect. Immun*. **68,** 5881–5888.

Kato, H., Kato, N., Katow, S., Maegawa, T., Nakamura, S. and Lyerly, D. (1999). Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol. Lett*. **175,** 197–203.

Kelly, C.P. and LaMont, J.T. (1998). *Clostridium difficile* infection. *Annu. Rev. Med*. **49,** 375–390.

Kelly, C.P., Pothoulakis, C. and LaMont, J.T. (1994). *Clostridium difficile* colitis. *New England J. Med*. **330,** 257–262.

Kink, J.A. and Williams, J.A. (1998). Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. *Infect. Immun*. **66,** 2018–2025.

Klapproth, J.-M., A., Scaletsky, I.C.A., McNamara, B.P., Lai, L.-C., Malstrom, C., James, S.P. and Donnenberg, M.S. (2000). A large toxin from pathogenic *Escherichia coli* strains that inhibits lymphocyte activation. *Infect. Immun*. **68,** 2148–2155.

Kozma, R., Ahmed, S., Best, A. and Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol*. **15,** 1942–1952.

Krivan, H.C., Clark, G.F., Smith, D.F. and Wilkins, T.D. (1986). Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. *Infect. Immun*. **53,** 573–581.

Kuijper, E.J., de Weerdt, J., Kato, H., Kato, N., van Dam, A.P., van der Vorm, E.R., Weel, J., van Rheenen, C. and Dankert, J. (2001). Nosocomial outbreak of Clostridium difficile-associated diarrhea due to a clindamycin-resistant enterotoxin A-negative strain. *Eur. J. Clin. Microbiol. Infect. Dis*. **20,** 528–534.

Larsen, R.D., Rivera-Marrero, C.A., Ernst, L.K., Cummings, R.D. and Lowe, J.B. (1990). Frameshift and nonsense mutations in a human genomic sequence homologous to a murine UDP-Gal:β-$_D$-Gal(1, 4)-$_D$-GlcNAc α(1, 3)-galactosyltransferase cDNA. *J. Biol. Chem*. **265,** 7055–7061.

Linseman, D.A., Hofmann, F. and Fisher, S.K. (2000). A role for the small molecular weight GTPases, Rho and Cdc42, in muscarinic receptor signaling to focal adhesion kinase. *J. Neurochem*. **74,** 2010–2020.

Liu, T.S., Musch, M.W., Sugi, K., Walsh-Reitz, M.M., Ropeleski, M.J., Hendrickson, B.A., Pothoulakis, C., LaMont, J.T. and Chang, E.B. (2003). Protective role of HSP72 against *Clostridium difficile* toxin A-induced intestinal epithelial cell dysfunction. *Am. J. Physiol. Cell Physiol*. **284,** C1073–C1082.

Lyerly, D.M., Saum K.E., MacDonald D.K. and Wilkins T.D. (1985). Effects of *Clostridium difficile* given intragastrically to animals. *Infect. Immun*. **47,** 349–352.

Lyerly, D.M., Phelps, C.J., Toth, J. and Wilkins, T.D. (1986). Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. *Infect. Immun*. **54,** 70–76.

Lyerly, D.M., Barroso, L.A., Wilkins, T.D., Depitre, C. and Corthier, G. (1992). Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect. Immun*. **60,** 4633–4639.

Mackay, D.J.G. and Hall, A. (1998). Rho GTPases. *J. Biol. Chem*. **273,** 20685–20688.

Maegawa, T., Karasawa, T., Ohta, T., Wang, X., Kato, H., Hayashi, H., Nakamura, S. (2002). Linkage betwen toxin production and purine biosynthesis in *Clostridium difficile. J. Med. Microbiol*. **51,** 34–41.

Mahida, Y.R., Makh, S., Hyde, S., Gray, T. and Borriello, S.P. (1996). Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: Induction of interleukin 8 production and apoptosis after cell detachment. *Gut* **38,** 337–347.

Mani, N. and Dupuy, B. (2001). Regulation of toxin synthesis in *Clostridium difficile* by an alternative RNA polymerase sigma factor. *PNAS* **98,** 5844–5849.

Mani, N., Lyras, D., Barroso, L., Howarth, P., Wilkins, T., Rood, J.I., Sonenshein, A.L. and Dupuy, B. (2002). Environmental response and autoregulation of *Clostridium difficile* TxeR, a sigma factor for toxin gene expression. *J. Bacterial*. **184,** 5971–5978.

Mantyh, C.R., McVey, D.C. and Vigna, S.R. (2000). Extrinsic surgical denervation inhibits Clostridium difficile txon A-induced enteritis in rats. *Neuroscience Lett*. **292,** 95–98.

Martinez, R.D. and Wilkins T.D. (1992). Comparison of *Clostridium sordellii* toxins HT and LT with toxins A and B of *C.difficile. J.Med.Microbiol*. **36,** 30–36.

Mehlig, M., Moos, M., Braun, V., Kalt, B., Mahony, D.E. and Eichel-Streiber, C.V. (2001). Variant toxin B and a functional toxin A produced by *Clostridium difficile* C34. *FEMS Microbiol. Lett*. **198,** 171–176.

Mesmin, B., Robbe, K., Geny, B., Luton, F., Brandolin, G., Popoff, M. and Antonny, B. (2004). A phospatidylserine-binding site in the cytosolic fragment of Clostridium sordellii TcsL facilitates glucosylation of membrane-bound Rac and is required for cytotoxicity. *J. Bol. Chem*. **279,** 49876–49882.

Moncrief, J.S., Barroso, L.A. and Wilkins T.D. (1997). Positive regulation of *Clostridium difficile* toxins. *Infect Immun*. **65,** 1105–1108.

Moncrief and Wilkins, (2000). Genetics of *Clostridium difficile* toxins. In: *Clostridium Difficile*. (eds.) Aktories, K. & Wilkins, T.D. *Curr Top Microbiol. Immunol*. **250,** 35–54.

Moore, R., Pothoulakis, C., LaMont, J.T., Carlson, S. and Madara, J.L. (1990). C. difficile toxin A increases intestinal permeability and induces $Cl^-$. *Am. J. Physiol*. **259,** G165–G172.

Neunlist, M., Barouk, J., Michel, K., Just, I., Oreshkova, T., Schemann, M. and Galmiche, J.P. (2003). Toxin B of Clostridium difficile activates human VIP submucosal neurons in part via an Il-1beta-dependent pathway. *Am. J. Physiol. Gastrointest. Liver Physiol*. **285,** G1028–G1036.

Nusrat, A., Giry, M., Turner, J.R., Colgan, S.P., Parkos, C.A., Carnes, D., Lemichez, E., Boquet, P. and Madara, J.L. (1995). Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. *Proc. Natl. Acad. Sci. USA* **92,** 10629–10633.

Nusrat, A., Von Eichel-Streiber, C., Turner, J.R., Verkade, P., Madara, J.L. and Parkos, C.A. (2001). *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infect. Immun*. **69,** 1329–1336.

Osgood, D.P., Wood, N.P. and Sperry, J.F. (1993). Nutritional aspects of cytotoxin production by *Clostridium difficile. Appl. Environ. Microbiol*. **59,** 3985–3988.

Petit, P., Breard, J., Montalescot, V., El Hadj, N.B., Levade, T., Popoff, M. and Geny, B. (2003). Lethal toxin from Clostridium sordellii induces apoptotic cell death by disruption of mitochondrial homeostasis in HL-60 cells. *Cell. Microbiol*. **5,** 761–771.

Pfeifer, G., Schirmer, J., Leemhuis, J., Busch, C., Meyer, D.K., Aktories, K. and Barth, H. (2003). Cellular uptake of *Clostridium difficile* toxin B: translocation of the N-terminal catalytic domain into the cytosol of eukaryotic cells. *J. Biol. Chem*. **278,** 44535–44541.

Phelps, C.J., Lyerly, D.L., Johnson, J.L. and Wilkins, T.D. (1991). Construction and expression of the complete *Clostridium difficile* toxin A gene in *Escerichia coli. Infect. Immun*. **59,** 150–153.

Popoff, M.R. *et al*. (1996). Ras, Rap, and Rac small GTP-binding proteins are targets for Clostridium sordellii lethal toxin glucosylation. *J. Biol. Chem*. **271,** 10217–10224.

Pothoulakis, C., Sullivan, R., Melnick, D.A., Triadafilopoulos, G., Gadenne, A.-S., Meshulam, T. and LaMont, J.T. (1988). *Clostridium difficile* toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. *J. Clin. Invest*. **81,** 1741–1745.

Pothoulakis, C., LaMont, J.T., Eglow, R., Gao, N., Rubins, J.B., Theoharides, T.C. and Dickey, B.F. (1991). Characterizing of rabbit ileal receptors for *Clostridium difficile* toxin A. *J. Clin. Invest*. **88,** 119–125.

Pothoulakis, C. *et al*. (1996). Rabbit sucrase-isomaltase contains a functional intestinal receptor for *Clostridium difficile* toxin A. *J. Clin. Invest*. **98,** 641–649.

Pothoulakis, C., Castagliuolo, I. and LaMont, J.T. (1998). Nerves and intestinal mast cells modulate responses to enterotoxins. *News. Physiol. Sci*. **13**, 58–63.

Pothoulakis, C. and LaMont, J.T. (2001). Microbes and microbial toxins: Paradigms for microbial-mucosal interactions. *Am. J. Physiol. Gastrointest. Liver Physiol*. **280,** G178–G183.

Poxton, I.R., McCoubrey, J. and Blair, G. (2001). The pathogenicity of Clostridium difficile. *Clin. Microbiol. Infect*. **7,** 421–427.

Prepens, U., Just, I., Von Eichel-Streiber, C. and Aktories, K. (1996). Inhibition of FceRI-mediated activation of rat basophilic leukemia cells by *Clostridium difficile* toxin B (monoglucosyltransferase). *J. Biol. Chem*. **271,** 7324–7329.

Qa'Dan, M., Spyres, L.M. and Ballard, J.D. (2000). pH-induced conformational changes in *Clostridium difficile* toxin B. *Infect. Immun*. **68,** 2470–2474.

Qa'Dan, M., Ramsey, M., Daniel, J., Spyres, L.M., Safiejko-Mroczka, B., Ortiz-Leduc, W. and Ballard, J.D. (2002). Clostridium difficile toxin B activates dual caspase-dependent and caspase-independent apoptosis in intoxicated cells. *Cell. Microbiol*. **4,** 425–434.

Raffestin, S., Dupuy, B., Marvaud, J.C. and Popoff, M.R. BotR/A and TetR are alternative RNA polymerase sigma factors controlling the expression of the neurotoxin and associated protein genes in *Clostridium botulinum* type A and *Clostridium tetani. Mol. Microbiol*. **55,** 235–249.

Ravizzola, G., Manca, N., Dima, F., Signorini, C., Garrafa, E. and Turano, A. (1998). Isolation of a *Clostridium* exotoxin producer other than *Clostridium difficile* from a patient with diarrhea. *J. Clin. Microbiol*. **36,** 2396.

Ridley, A.J. (2001). Rho family proteins: coordinating cell responses. *Trends Cell Biol*. **11,** 471–477.

Ridley, A.J. (2000). Rho. In: *GTPases* (ed. A.Hall) pp. 89–136 Oxford University Press, Oxford.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70,** 401–410.

Riegler, M. *et al*. (1995). *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium *in vitro. J. Clin. Invest*. **95,** 2004–2011.

Rupnik, M., Avesani, V., Janc, M., Eichel-Streiber, C.v. and Delmee, M. (1998). A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J. Clin. Microbiol*. **36,** 2240–2247.

Rupnik, M., Braun, V., Soehn, F., Janc, M., Hofstetter, M., Laufenberg-Feldmann, R. and Eichel-Streiber, C.v. (1997). Characterization of polymorphisms in the toxin A and B genes of *Clostridium difficile. FEMS Microbiol. Lett*. **148,** 19–202.

Rupnik, M., Brazier, J.S., Duerden, B.I., Grabnar, M. and Stubbs, S.L.J. (2001). Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiology* **147,** 439–447.

Rupnik, M., Kato, N., Grabnar, M. and Kato, H. (2003). New types of toxin A-negative, toxin B-positive strains among Clostridium difficile isolates from Asia. *J. Clinic. Microbiol*. **41,** 1118–1125.

Rupnik, M., Dupuy, B., Fairweather, N.F., Gerding, D.N., Johnson, S., Just, I., Lyerly, D.M., Popoff, M.R., Rood, J.I., Sonenshein, A.L., Thelestam, M., Wren, B.W., Wilkins, T.D. and Eichel-Streiber C.v. (2005a). Revised nomenclature of *Clostridium difficile* toxins and associated genes. *J. Med. Microbiol*. **54,** 113–117.

Rupnik M., Pabst, S., Rupnik, M., Eichel-Streiber, C.v., Urlaub, H., Söling, H.-D. (2005b). Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. *Microbiology*, **151,** 199–208.

Sambol, S., Merrigan, M., Lyerly, D., Gerding, D. and Johnson, S. (2000). Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. *Infect. Immun*. **68,** 5480–5487.

Sambol S.P., Tang J.K., Merrigan M.M., Johnson S. and Gerding D.N. (2001). Infection of hamsters with epidemiologically important strains of Clostridium difficile. *J. Infect. Dis*. **183,** 1760–1766.

Savidge, T.C., Pan, W.H., Newman, P., O'Brien, M., Anton, P.M. and Pothoulakis, C. (2003). *Clostridium difficile* toxin B is an inflammatory enterotoxin in human intestine. *Gastroenterology* **125,** 413–420.

Schiavo, G. and van der Goot, F.G. (2001). The bacterial toxin toolkit. *Nat. Mol. Cell Biol*. **2,** 530–537.

Schmidt, M., Rümenapp, U., Bienek, C., Keller, J., Von Eichel-Streiber, C. and Jakobs, K.H. (1996). Inhibition of receptor signaling to phospholipase D by *Clostridium difficile* toxin B—role of Rho proteins. *J. Biol. Chem*. **271,** 2422–2426.

Schmidt, M., Vo, M., Thiel, M., Bauer, B., Grannass, A., Tapp, E., Cool, R.H., De Gunzburg, J., Von Eichel-Streiber, C. and Jakobs, K.H. (1998). Specific inhibition of phorbol ester-stimulated phospholipase D by *Clostridium sordellii* lethal toxin and *Clostridium difficile* toxin B-1470 in HEK-293 cells. *J. Biol. Chem*. **273,** 7413–7422.

Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E. and Aktories, K. (1998). Glucosylation and ADP-ribosylation of Rho proteins—effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry* **37,** 5296–5304.

Selzer, J., Hofmann, F., Rex, G., Wilm, M., Mann, M., Just, I. and Aktories, K. (1996). *Clostridium novyi* a-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. *J. Biol. Chem*. **271,** 25173–25177.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W. and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287,** 1037–1040.

Shao, F., Merritt, P.M., Bao., Z., Innes, R.W. and Dixon, J.E. (2002). A *Yersinia* effector and a Pseudomonas avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. *Cell* **109,** 575–588.

Smith, J.A., Cooke, D.L., Hyde, S., Borriello, S.P. and Long, R.G. (1997). *Clostridium difficile* toxin A binding to human intestinal epithelial cells. *J. Med. Microbiol*. **46,** 953–958.

Soehn, F., Wagenknecht-Wiesner, A., Leukel, P., Kohl, M., Weidmann, M., Eichel-Streiber, C.v. and Braun, V. (1998). Genetic rearrangements in the pathogenicity locus of *Clostridium difficile* strain 8864 – implications for transcription, expression, and enzymatic activity of toxins A and B. *Mol. Gen. Genet*. **258,** 222–232.

Song, K.P., Ow, S.E., Chang, S.Y. and Bai, X.L. (1999). Sequence analysis of a new open reading frame located in the pathogenicity locus of *Clostridium difficile* strain 8864. *FEMS Microbiol. Lett*. **180,** 241–248.

Spigaglia, P. and Mastrantonio, P. (2002). Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J. Clin. Microbol*. **40,** 3470–3475.

Spyres, L.M., Qa'Dan, M., Meader, A., Tomasek, J.J., Howard, E.W. and Ballard, J.D. (2001). Cytosolic delivery and characterization of the TcdB glucosylating domain by using heterologous protetin fusion. *Infect. Immun*. **69,** 599–601.

Stevens, M.P., Roe, A.J., Vlisidou, I. van Diemen, P.M., LaRagione, R.M., Best, A., Woodward, M.J., Gally, D.L. and Wallis, T.S. (2004). Mutation of *toxB* and a truncated version of the *efa-1* gene in *Escherichia coli* O157: H7 influences the expression and secretion of locus of enterocyte effecement-encoded proteins but not intestinal colonization in calves or sheep. *Infect. Immun*. **72,** 5402–5411.

Stoddart, B. and Wilcox, M.H. (2002). Clostridium difficile. *Curr. Opin. Infect. Dis*. **15,** 513–518.

Subauste, M.C., Von Herrath, M., Benard, V., Chamberlain, C.E., Chuang, T.H., Chu, K., Bokoch, G.M. and Hahn, K.M. (2000). Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. *J. Biol. Chem*. **275,** 9725–9733.

Takai, Y., Sasaki, T. and MAtozaki, T. (2001). Small GTP-binding proteins. *Physiol. Rev*. **81,** 153–208.

Tan, K.S., Wee, B.Y. and Song, K.P. (2001). Evidence for holin function of *tcdE* gene in the pathogenicity of *Clostridium difficile*. *J. Med. Microbiol*. **50,** 613–619.

Tang-Feldman, Y.J., Ackermann, G., Henderson, J.P., Silva Jr, J. and Cohen, S.H., (2002). One-step cloning and expression of *Clostridium difficile* toxin B gene (*tcdB*). *Mol. Cell. Probes* **16,** 179–183.

Teneberg, S., Lönnroth, I., López, J.F.T., Galili, U., Halvarsson, M.Ö., Ångström, J. and Karlsson, K.A. (1996). Molecular mimicry in the recognition of glycosphingolipids by Galα3Galβ4GlcNAcβ-binding *Clostridium difficile* toxin A, human natural anti α-galactosyl IgG and the monoclonal antibody Gal-13: characterization of a binding-active human glycosphingolipid, non-identical with the animal receptor. *Glycobiology* **6,** 599–609.

Thelestam, M., Chaves-Olarte, E., Moos, M. and Eichel-Streiber, C.v. (1999). Clostridial toxins acting on the cytoskeleton. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. Alouf, J.E., Freer, J.H.) Academic Press, London.

Thelestam, M. and Chaves-Olarte, E. (2000). Cytotoxic effects of the Clostridium difficile toxins. *Curr. Top. Microbiol. Immunol*. **250,** 85–96.

Torres, J.F. (1991). Purification and characterization of toxin B from strain of *Clostridium difficile* hat does not produce toxin A. *J. Med. Microbiol*. **35,** 40–44.

Tucker, K.D. and Wilkins, T.D. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. *Infect. Immun*. **59,** 73–78.

Unligil, U.M. and Rini, J.M. (2000). Glycosyltransferase structure and mechanism. *Curr. Opin. Struct. Biol*. **10,** 510–517.

Unligil, U.M., Zhou, S., Yuwaraj, S., Sarkar, M., Schachter, H. and Rini, J.M. (2000). X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. *EMBO J*. **19,** 5269–5280.

Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes & Development* **11,** 2295–2322.

Vetter, I.R., Hofmann, F., Wohlgemuth, S., Herrmann, C. and Just, I. (2000). Structural consequences of mono-glucosylation of

Ha-Ras by Clostridium sordellii lethal toxin. *J. Mol. Biol*. **301,** 1091–1095.

von Eichel-Streiber, C. and Sauerborn, M. (1990). Clostridium difficile toxin A carries a C-terminal structure homologous to the carbohydrate binding region of streptococcal glycosyltransferase. *Gene* **96,** 107–113.

von Eichel-Streiber, C., Boquet, P., Sauerborn, M. and Thelestam, M. (1996). Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. *TIM* **4,** 375–382.

von Eichel-Streiber, C., Habermann, E., Weidmann, M. and Hofmann, F. (1994). Genetical analysis of the large clostridial cytotoxins – a comparison of Clostridium novyi, C. sordellii, and C. difficile toxins. In: *Bacterial Protein Toxins* (Eds. Freer *et al*., ), pp. 51–59, Zbl.Bakt. Suppl. 24, Gustav Fischer, Stuttgart.

von Eichel-Streiber, C., Harperath, U., Bosse, D., Hadding, U. (1987). Purification of two high molecular mass toxins of *Clostridium difficile* which are antigenically related. *Microbial. Pathogen*. **2,** 307–318.

von Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartingen, S., Shulze, J. and Sauerborn M. (1992). Comparative sequence analysis of the *Clostridium difficile* toxins A and B. *Mol. Gen. Genet.* **233,** 260–268.

von Eichel-Streiber, C., Laufenberg-Feldmann, R., Sartringen, S., Schulze, J. and Sauerborn, M. (1990). Cloning of *Clostridium difficile* toxin B gene and demonstration of high N-terminal homology between toxin A and B. *Med. Microbiol. Immunol*. **179,** 271–279.

von Eichel-Streiber, C., Meyer zu Heringdorf, D., Habermann E. and Sartringen, S. (1995). Closing in on the toxic domain through analysis of a variant *Clostridium difficile* cytotoxin B. *Mol. Microbiol*. **17,** 313–321.

von Eichel-Streiber, C., Zec-Pirnat, I., Grabnar, M. and Rupnik, M. (1999). A nonsense mutation abrogates production of a functional enterotoxin A in *Clostridium difficile* toxinotype VIII strains of serogroup F and X. *FEMS Microbiol. Lett*. **178,** 163–168.

von Eichel-Streiber, C., Sauerborn, M. and Kuramitsu, H.K. (1992). Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and *Streptcoccus mutans* glucosyltransferases. *J. Bacteriol*. **174,** 6707–6710.

Voth, D.E., Qa'Dan, M., Hamm, E.E., Pelfrey, J.M. and Ballard, J.D. (2004). Clostridium sordellii lethal toxin is maintained in a multimeric protein complex. *Infect. Immun*. **72,** 3366–3372.

Wagenknecht-Wiesner, A., Weidman, M., Braun, V., Leukel, P., Moos, M. and Eichel-Streiber, C.v. (1997). Delineation of the catalytic domain of *Clostridium difficile* toxin B-10463 to an enzymatically active N-terminal 467 amino acid fragment. *FEMS Microbiol. Lett*. **152,** 109–116.

Warny, M., Keates, A.C., Keates, S., Castagliuolo, I., Zacks, J.K., Aboudola, S., Qamar, A., Pothoulakis, C., LaMont, J.T. and Kelly, C.P. (2000). p38MAP kinase activation by Clostridum difficile toxin A mediates monocytes necrosis, IL-8 production, and enteritis. *J. Clin. Invest*. **105,** 1147–1156.

Wettschureck, N. and Offermanns, S. (2002). Rho/Rho-kinase mediated signaling in physiology and pathophysiology. *J. Mol. Med*. **80,** 629–638.

Wiggins, C.A.R. and Munro, S. (1998). Activity of the yeast *MNN1* a-1, 3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. *Proc. Natl. Acad. Sci. USA* **95,** 7945–7950.

Wren, B.W. (1991). A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. *Mol. Microbiol*. **5,** 797–803.

Yamakawa, K., Kamiya, S., Meng, X.Q., Karasawa, T. and Nakamura, S. (1994). Toxin production by *Clostridium difficile* in a defined medium with limited amino acids. *J. Med. Microbiol*. **41,** 319–323.

Yamakawa, K., Karasawa, T., Ikoma, S. and Nakamura, S. (1996). Enhacement of *Clostridium difficile* toxin production in biotin-limited conditions. *J. Med. Microbiol*. **44,** 111–114.

Zhang, Z., Vuori, K., Wang, H.-G., Reed, J.C. and Ruoslahti, E. (1996). Integrin activation by R-ras. *Cell* **85,** 61–69.

Zhao, Y., Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (cpe) of *Clostridium perfringens*. *J. Bacteriol*. **180,** 136–142.

Zohn, I.M., Campbell, S.L., Khosravi-Far, R., Rossman, K.L. and Der, C.J. (1998). Rho family proteins and Ras transformation: the RHOad less traveled gets congested. *Oncogene* **17,** 1415–1438.

CHAPTER

# 22

# *Pasteurella multocida* toxin

*Brenda A. Wilson and Mengfei Ho*

The major virulence factor of *Pasteurella multocida* responsible for atrophic rhinitis, pneumonia-like respiratory disease, and dermonecrosis is a monomeric 1285 amino acid protein toxin (PMT) produced primarily by capsular type D, some capsular type A, and a few non-typable strains. The *toxA* gene that encodes for PMT is located on a prophage pathogenicity island. While it is not known how PMT is released from the bacteria, purified PMT alone is sufficient to reproduce all the symptoms of atrophic rhinitis in swine and pneumonia-like disease in rabbits. Antibodies generated against PMT are protective toward atrophic rhinitis in animals. Experiments to define the molecular mechanisms of PMT action over the past 15 years have revealed that PMT binds to ganglioside-type receptors and enters mammalian cells via receptor-mediated endocytosis, where it then acts intracellularly to activate $G_q$-protein coupled signal transduction, Rho-protein-dependent actin cytoskeletal rearrangements, and mitogenesis. This chapter reviews what is currently known about the structure and molecular action of PMT, its role in pathogenesis, and its interaction with and effects on mammalian cells. Examples are also provided of the use of PMT as a powerful tool to study mitogenic, $G_q$-protein-, and Rho-protein-dependent signaling mechanisms.

## INTRODUCTION

### *Pasteurella multocida* and atrophic rhinitis

#### *Atrophic rhinitis and pasteurellosis*

Atrophic rhinitis is a disease of domestic and wild animals that is characterized by atrophy of the nasal turbinate bones, manifested by shortening, wrinkling, and twisting of the snout, sneezing, snuffling, nasal, discharge, teary eyes, and growth retardation. Depending on the severity of the disease, pathological changes can be mild and restricted to the snout with no overt clinical signs other than shrinkage of the ventral turbinates to severe and progressive disease with complete loss of all turbinate bone structures, septum deviation, respiratory distress, lung lesions, and generalized small stature and slow growth.

Atrophic rhinitis is an economically significant disease in swine, cattle, sheep, and rabbits, but it can affect other animals, such as small and large ruminants, poultry, cats, and dogs, as well as humans, and is therefore considered a zoonotic disease. In swine and rabbits, the primary disease manifestation is atrophic rhinitis (DiGiacomo *et al*., 1989; Foged, 1992), while in cattle, rabbits, and other animals other symptoms may be more pronounced, including respiratory distress or pneumonia (also referred to as pasteurellosis) and abscess formation (Kielstein, 1986; Deeb *et al*., 1990; DiGiacomo *et al*., 1991). An experimental mouse model of progressive atrophic rhinitis similar to the swine disease has been developed (Jordan and Roe, 2004).

#### *Atrophic rhinitis in swine*

Infection with *Bordetella bronchiseptica* or *Pasteurella multocida* alone or in combination can result in atrophic rhinitis in swine (reviewed in Chanter, 1990; Foged, 1992). After the first report in 1956 that intranasal inoculation of *B. bronchiseptica* could induce atrophic rhinitis in piglets (Switzer, 1956), it was generally believed that only *B. bronchispetica* could cause atrophic rhinitis. However, in the early 1980s, the correlation between severe clinical and pathological

symptoms of turbinate atrophic rhinitis and toxigenic isolates of *P. multocida* was firmly established (Kielstein, 1986; Foged, 1992). It is now well recognized that *B. bronchiseptica* alone causes a mild, transient form of atrophic rhinitis (Tornoe and Nielsen, 1976; Pedersen and Barfod, 1981; Rutter, 1981; Rutter *et al*., 1982; Rutter, 1985; Roop *et al*., 1987; Magyar *et al*., 1988), and while widespread, it has little impact on the productivity and health status of the animal. On the other hand, *P. multocida* infection is associated with moderate to severe progressive atrophic rhinitis (see Figure 22.1), which is also accompanied by growth retardation, and can have a significant health and economic impact (Pedersen and Barfod, 1981; Elling and Pedersen, 1985; Pedersen *et al*., 1988). Furthermore, it has been found that prior exposure to or co-infection with *B. bronchiseptica* predisposes piglets to colonization by *P. multocida* and to enhanced severity and progression of atrophic rhinitis and growth retardation (Harris and Switzer, 1968; Pedersen and Barfod, 1982; Rutter, 1983; Pedersen and Elling, 1984; de Jong and Akkermans, 1986; Nakai *et al*., 1986; Chanter *et al*., 1989; van Diemen *et al*., 1994a; Ackermann *et al*., 1996). Other predisposing infections can also exacerbate atrophic rhinitis by *P. multocida* (Brockmeier *et al*., 2001). In studies using non-toxigenic strains of *B. bronchiseptica* (i.e., lacking the *Bordetella* dermonecrotic toxin), it was found that colonization by toxigenic *P. multocida* was significantly reduced and hence atrophic rhinitis was much less severe (Magyar *et al*., 1988), suggesting that the toxin from *Bordetella* is a contributing factor for colonization.

**FIGURE 22.1** Atrophic rhinitis in swine. Shown are transverse sections of the nasal cavity of pigs exhibiting pathological changes of atrophic rhinitis ranging from mild (top panel) to moderate (middle panel) to severe (bottom panel) caused by infection with *Pasteurella multocida*. Photos courtesy of the University of Illinois Veterinary Diagnostics Laboratory, Urbana, Illinois.

### *Pasteurellosis in rabbits*

*P. multocida* is the primary etiological agent of atrophic rhinitis and respiratory disease (pasteurellosis or "snuffles") in rabbits (DiGiacomo *et al*., 1983; DiGiacomo *et al*., 1990; Deeb and DiGiacomo, 2000). Type A:12 strains are the most common in rabbits in the U.S., but A:3 and other A and D serotypes are found, with more severe disease being associated with A:3 and D strains (DiGiacomo *et al*., 1989; DiGiacomo *et al*., 1990; DiGiacomo *et al*., 1991). As for swine, co-infection with *B. bronchiseptica* increases the ability of *P. multocida* to colonize and cause disease (Deeb *et al*., 1990).

## Role of PMT in pathogenesis

Shortly after the demonstration that persistent turbinate atrophy could be induced by toxigenic strains of *P. multocida*, cell-free extracts from these strains were used to identify and isolate the toxin responsible for the symptoms of atrophic rhinitis (Il'ima and Zasukhin, 1975; Chanter *et al*., 1986). Early descriptions of assays used to monitor the activity of the toxin preparations included induction of turbinate atrophy after intranasal exposure in piglets (Elling and Pedersen, 1985; Chanter *et al*., 1986; de Jong and Akkermans, 1986; Dominick and Rimler, 1986; Elias *et al*., 1986; Foged *et al*., 1987; Dominick and Rimler, 1988; Kamp and Kimman, 1988; Elias *et al*., 1990); induction of atrophic rhinitis and other organ lesions after intramuscular, intraperitoneal, or intravenous injection (Rutter and Mackenzie, 1984; Chanter *et al*., 1986; de Jong *et al*., 1986; Cheville *et al*., 1988; Kamp and Kimman, 1988; Cheville and Rimler, 1989; Elias *et al*., 1990); induction of atrophic rhinitis and pneumonia in rabbits (Foged *et al*., 1987; Chrisp and Foged, 1991); guinea pig or rat dermonecrotic lesion assays (Nakai

*et al*., 1984b; Foged *et al*., 1987; Kamp *et al*., 1987; Elling *et al*., 1988); BALB/c mouse lethality assay (Rutter, 1983; Nakai *et al*., 1984b; Foged *et al*., 1987; Kamp *et al*., 1987); and cell culture assays of cytopathicity (Pennings and Storm, 1984; Rutter and Luther, 1984; Kimman *et al*., 1987; Chrisp and Foged, 1991). Results from these studies demonstrated that purified PMT alone could induce turbinate atrophy and other symptoms of disease, as well as lethality, dermonecrotic, cytopathic, mitogenic, and other biological effects. The primary systemic effects of PMT intoxication observed in laboratory animals were liver necrosis (Cheville *et al*., 1988; Cheville and Rimler, 1989; Lax and Chanter, 1990; Chrisp and Foged, 1991), spleen atrophy (Nakai *et al*., 1984a; Nakai *et al*., 1984b; Foged, 1988; Chrisp and Foged, 1991), hydronephrosis of the kidneys (Chanter *et al*., 1986; Lax and Chanter, 1990), pneumonia (Chrisp and Foged, 1991), and growth retardation (van Diemen *et al*., 1994a; Ackermann *et al*., 1996).

PMT appears to cause nasal turbinate atrophy through disruption of normal intramembranous bone biogenesis and degradation processes, mediated by the bone generating osteoblasts and macrophage-like osteoclasts, respectively (Kimman *et al*., 1987; Mullan and Lax, 1996; Mullan and Lax, 1998). PMT stimulates osteoclastic bone resorption *in vitro* (Felix *et al*., 1992; Mullan and Lax, 1996; Gwaltney *et al*., 1997; Mullan and Lax, 1998), stimulates the differentiation of preosteoclasts into osteoclasts (Martineau-Doize *et al*., 1993; Jutras and Martineau-Doize, 1996), and promotes osteoclast proliferation leading to bone resorption *in vivo* (Martineau-Doize *et al*., 1993). *In vitro*, PMT inhibits bone regeneration by osteoblasts (Sterner-Kock *et al*., 1995; Mullan and Lax, 1996; Mullan and Lax, 1998), as well as osteoblast differentiation (Harmey *et al*., 2004).

While atrophic rhinitis is strongly associated with infection by toxigenic capsular type D and A strains of *P. multocida*, the correlation between the presence of both atrophic rhinitis and pneumonic pasteurellosis is not always consistent with the presence of toxigenic strains. Unlike the case for rabbits (DiGiacomo *et al*., 1989; Chrisp and Foged, 1991; Suckow *et al*., 1991; DiGiacomo *et al*., 1993), pneumonic pasteurellosis in pigs, cattle, and poultry is most commonly associated with non-toxigenic capsular type A strains (Adler *et al*., 1999; Dowling *et al*., 2002; Davies *et al*., 2003; Davies *et al*., 2004; Dowling *et al*., 2004). However, there are reports of toxigenic capsular type A strains of *P. multocida* associated with pneumonic pasteurellosis and atrophic rhinitis (Kielstein and Elias, 1985; de Jong *et al*., 1986; Sakano *et al*., 1992). Indeed, immunization with bacterin-toxoid vaccine of *B. bronchiseptica* and toxigenic capsular type D *P. multocida* provides protection against challenge with both toxigenic serotype A and D strains (Sakano *et al*., 1997).

## Immunity and vaccine development

The importance of the toxin in *P. multocida* pathogenesis and its potential as a vaccine target was first demonstrated by protection against atrophic rhinitis in pigs challenged with *B. bronchiseptica* and toxigenic *P. multocida* through vaccination of pregnant sows with filtrates from cultures of toxigenic *P. multocida* (Pedersen and Barfod, 1982). PMT does not appear to be released sufficiently by the bacteria such that it is efficiently presented to the immune system during infection. Naturally occurring atrophic rhinitis is characterized by a lack of immune response against PMT (van Diemen *et al*., 1994b; van Diemen *et al*., 1996). Initial studies using only bacterin of toxigenic *P. multocida* resulted frequently in low levels of toxin neutralizing antibodies (Rutter *et al*., 1984; Rutter, 1985; Chanter *et al*., 1989; Thurston *et al*., 1991; Thurston *et al*., 1992), even though immunization with toxoid alone afforded protection (Pedersen and Barfod, 1982; Foged *et al*., 1989; Frymus *et al*., 1989; Foged, 1991; Nielsen *et al*., 1991; Pettit *et al*., 1993b). Parenteral immunization of pigs, rabbits, and mice with formaldehyde-treated crude (Pedersen and Barfod, 1982; Nakai *et al*., 1984a; Nakai *et al*., 1984b; Bording and Foged, 1991) or purified (Foged *et al*., 1989; Frymus *et al*., 1989; Chanter and Rutter, 1990; Bording and Foged, 1991; Foged, 1991) preparations of PMT have been shown to produce protective, neutralizing antibodies against PMT effects. In addition, colonization of toxigenic *P. multocida* in piglets was reduced in piglets inoculated with porcine anti-PMT serum before or after exposure to bacteria (Chanter and Rutter, 1990). Most current vaccine formulations now consist of PMT toxoid alone (Nielsen *et al*., 1991; Petersen *et al*., 1991; Jarvinen *et al*., 1998) or PMT toxoid in combination with toxigenic *P. multocida* and/or *B. bronchiseptica* bacterins (Sakano *et al*., 1997; Suckow, 2000; Magyar *et al*., 2002; Riising *et al*., 2002; Rajeev *et al*., 2003).

Although protective immunity can be induced by intranasal immunization with PMT toxoid in rabbits (Suckow *et al*., 1995), rabbits generally develop little immunity after natural infection, and high levels of humoral antibodies (IgG) are not associated with clearance of infection, but rather with chronic infection (Deeb *et al*., 1990; DiGiacomo *et al*., 1990; Zimmerman *et al*., 1992). Pigs treated with PMT likewise show lower IgG antibody responses toward PMT and other antigens, including limpet hemocyanin, ovalbumin, and tetanus toxoid (van Diemen *et al*., 1996). In another

study, a reduction in the humoral response to ovalbumin antigen was also observed in pigs challenged intranasally with toxigenic *P. multocida* compared to non-infected control animals, suggesting that PMT might have contributed to suppression of the immune response to antigen (Hamilton *et al*., 1998). A possible role for PMT as an immunomodulator in pathogenesis was further supported in a subsequent study that showed intranasal challenge with toxigenic *P. multocida* or with PMT-containing cell-free extracts caused a significant reduction in the levels of anti-ovalbumin IgG in both pigs and mice (Jordan *et al*., 2003).

## PMT CHARACTERIZATION

### PMT pathogenicity island

The gene encoding for PMT, named *toxA*, was cloned from the chromosomal DNA of *P. multocida* by three independent laboratories (Petersen and Foged, 1989; Buys *et al*., 1990; Kamps *et al*., 1990; Lax and Chanter, 1990). The deduced sequence of the 3,855-basepair open reading frame of *toxA* corresponded to a protein of 1285 amino acids and a predicted molecular mass of 146.3 kDa (Buys *et al*., 1990) or 146.5 (Petersen and Foged, 1989), with the molecular weight differences being attributed to the small number of amino acid changes in the sequences from the different isolates. Sequence analysis revealed that a ribosome-binding site preceded the start of the *toxA* open reading frame and that a stem loop followed the stop codon. There was no evidence for a signal sequence. The promoter region is different from that of an *E. coli* consensus promoter, yet expression under its own promoter in *E. coli* is constitutive and comparable to that found in *P. multocida* (Petersen and Foged, 1989; Lax and Chanter, 1990; Hoskins and Lax, 1996).

The *toxA* gene and its flanking region had a low G+C content (34.6%), which differed from the rest of the *P. multocida* genome (40.7%), as recently confirmed by whole genome sequencing of a non-toxigenic strain of *P. multocida* (May *et al*., 2001). When the chromosomal DNA was exhaustively digested with the restriction enzyme *Hpa*II, which recognizes the 4-basepair GGCC sequence, the *toxA* gene was located on a prominent 15-kilobasepair *Hpa*II DNA fragment (Lax and Chanter, 1990). This suggested that the gene might reside on a transmissible element, presumably a prophage (Andresen, 1989; Buys *et al*., 1990; Lax *et al*., 1990; Petersen, 1990). In support of this possibility, a set of 24 bacteriophages, recovered after mitomycin C treatment of isolates associated with atrophic rhinitis, was used to develop an epidemiological typing system to discriminate between toxigenic and non-toxigenic *P. multocida* strains (Nielsen and Rosdahl, 1990).

Recently, further DNA sequencing of the region flanking *toxA* revealed the presence of additional genes with similarity to bacteriophage tail protein genes and a bacteriophage anti-repressor, as well as a restriction/modification system (Pullinger *et al*., 2004), supporting that *toxA* was located on a prophage. Analysis also indicated that the bacteriophage had integrated into a conserved tRNA-Leu-3 gene in the chromosome. The presence of a bacteriophage was also observed in the spent medium from *P. multocida* culture induced with mitomycin C, and hybridization indicated that the bacteriophage contained the *toxA* gene (Pullinger *et al*., 2004).

### PMT production and release

PMT does not have a signal sequence and appears to be mostly cell associated during *in vitro* culture, although small amounts can be detected in the culture filtrate after overnight incubation (Rutter and Luther, 1984; Nakai and Kume, 1987). Ultrastructural studies of toxigenic *P. multocida* showed that PMT was localized in the cytoplasm of the bacteria (Nakai *et al*., 1985; iDali *et al*., 1991). Although it is known that PMT is expressed under non-inducing conditions, it is possible that phage induction results in PMT release from cells during infection. The possibility that PMT expression is regulated by the phage life cycle has not been investigated.

### PMT protein purification and characterization

#### *Purification of native PMT*

Native PMT has been purified from cell-free extracts of *P. multocida* by a variety of methods, including ammonium sulfate precipitation, gel filtration, anion exchange chromatography, hydrophobic interaction chromatography, and preparative PAGE (Nakai *et al*., 1984b; Chanter *et al*., 1986; Foged *et al*., 1987; Kamp *et al*., 1987; Chrisp and Foged, 1991; Ward *et al*., 1998). Unlike most bacterial proteins, PMT is retained on anion exchange columns in neutral phosphate or Tris buffers and is eluted with relatively high salt concentrations (e.g., 400 mM NaCl). The final step of most purification protocols involves preparative PAGE, which yields highly purified toxin, but is also accompanied by significant loss in biological activity (Nakai *et al*., 1984b; Chanter *et al*., 1986; Foged *et al*., 1987; Kamp *et al*., 1987; Chrisp and Foged, 1991; Ward *et al*., 1998). Later methods for obtaining higher purification of the native protein from cell-free extracts involve affinity chromatography using monoclonal antibodies against PMT (Foged, 1988; Felix *et al*., 1992).

### *Purification of recombinant PMT*

The recombinant protein (rPMT) expressed in *E. coli* has been purified and extensively characterized by a number of laboratories (Petersen and Foged, 1989; Buys *et al*., 1990; Kamps *et al*., 1990; Lax and Chanter, 1990; Rozengurt *et al*., 1990; Wilson *et al*., 1999). rPMT was found to be indistinguishable in physical properties, biological activity, and antigenicity to that of native PMT. The only apparent difference is that the native PMT protein is posttranslationally modified at the N-terminus, whereas rPMT from *E. coli* is not. More recent strategies for obtaining highly purified rPMT preparations involve affinity purification using hexahistidine- or GST-tagged fusions, in which the affinity handle is then usually removed (Wilson *et al*., 1999; Pullinger *et al*., 2001; Orth *et al*., 2003).

### *Stability of PMT*

PMT can be completely inactivated by a number of protein-denaturing conditions, including by heating at 56˚C for 30–60 minutes or 70˚C for 5–10 minutes, or by treating at 20˚C with 0.37% formaldehyde for 1 hour or at 37˚C with 0.07% formaldehyde for 18 hours (Rutter, 1983; Nakai *et al*., 1984a; de Jong and Akkermans, 1986; Foged *et al*., 1987; Rozengurt *et al*., 1990; Foged, 1991). Unlike many bacterial protein toxins, PMT does not require or undergo additional processing, such as partial proteolytic cleavage at sensitive sites, in order to exhibit biological activity on cells. Compared to other bacterial protein toxins, full-length PMT is highly resistant to proteolytic cleavage by a number of proteases (Smyth *et al*., 1995; Smyth *et al*., 1999), but becomes susceptible at low pH, suggesting that reduced pH causes structural changes in the protein that make it more accessible to cleavage (Smyth *et al*., 1999). However, while this step may be important for translocation across cellular membranes, it is apparently not necessary for PMT to exhibit its intracellular activity, as evidenced by the rapid cellular response to PMT upon direct microinjection into cells (Wilson *et al*., 1997).

## PMT INTERACTION WITH AND ENTRY INTO MAMMALIAN CELLS

### Receptor binding

The initial step for internalization of many bacterial toxins is the binding to cell surface receptors, followed by receptor-mediated endocytosis into early endocytic vesicles. Once internalized, the toxin cannot be removed by extensive washing or neutralized by addition of antibodies. The cytotoxic effect of PMT on cultured fibroblasts could be blocked by polyclonal antiserum against the toxin, but only if the antiserum was added early, and not late, during the treatment with PMT (Rozengurt *et al*., 1990). PMT can act on multiple cells types, suggesting that the receptor(s) are ubiquitously expressed. Receptor-mediated binding and internalization of PMT conjugated to 20-nm colloidal gold particles into canine osteosarcoma and monkey kidney (Vero) cells was examined ultrastructurally (Pettit *et al*., 1993a). In these studies, preincubation of the cells with phospholipase C, trypsin, pronase, or neuraminidase had little effect on the association of gold-labeled PMT with the cells, but binding was blocked in a dose-dependent manner by addition of bovine brain mixed gangliosides, strongly suggesting that PMT interacts with ganglioside-type receptors (Pettit *et al*., 1993a). This finding was substantiated further by another study in which preincubation of PMT with the gangliosides GM1, GM2, or GM3 inhibited the PMT-mediated effects on 3T3 fibroblast cells (Dudet *et al*., 1996).

### Cellular uptake

Very little is known about the cellular uptake mechanisms of PMT. There is a notable lag period between initial exposure to PMT and observation of cellular effects, with earliest events usually seen after about one hour (Rozengurt *et al*., 1990, Wilson *et al*., 2000). Inhibition of endocytosis by incubation at 4˚C also prevented PMT-mediated effects on Swiss 3T3 fibroblast cells (Rozengurt *et al*., 1990).

Colloidal gold-labeled PMT was found bound at the cellular surface and in coated and non-coated membrane invaginations shortly after addition to cells, and in endocytic vesicles upon longer incubation (Pettit *et al*., 1993a). Fluorescein-labeled PMT was also used to observe cell binding and entry into bone cells (Gwaltney *et al*., 1997). However, when primary porcine bone marrow cell cultures were treated with fluorescein-labeled PMT, the fluorescence was found primarily on the surface and within the cytoplasm of lymphoid-like mononucleated cells, suggesting that toxin was translocated to the cytoplasm (Gwaltney *et al*., 1997). The difference in observations between these two studies is likely due to the gold particles being too large for transport or translocation to occur, whereas the fluorescein label did not hamper translocation.

After binding receptors at the cell surface, subsequent transport and/or membrane translocation of PMT from the endocytic vesicle to its target site of action appears to involve a low pH-dependent event, since addition of weak lysosomotrophic bases, such as methylamine, blocked PMT action (Rozengurt *et al*., 1990). Involvement of a low pH-dependent membrane

translocation event in PMT action was further supported by a recent study in which PMT cellular activity could be mimicked at the cell surface by a low pH pulse after PMT was bound to cells at 4°C in the presence of the endocytosis inhibitor bafilomycin A1 (Baldwin *et al*., 2004).

## MOLECULAR AND CELLULAR MECHANISMS OF PMT ACTION

### PMT effects on $G_q$-PLC signal transduction

#### *PMT and PLC signaling*

PMT stimulates hydrolysis of inositolphospholipids to increase the total intracellular content of inositol phosphates (Staddon *et al*., 1991a) and diacylglycerol (DAG) (Staddon *et al*., 1990); mobilization of intracellular $Ca^{2+}$ pools (Staddon *et al*., 1991a); and activation of protein kinase C (PKC)-dependent and independent phosphorylation (Staddon *et al*., 1990; Lacerda *et al*., 1996). These effects suggested the involvement of a cellular phosphatidylinositol ($PIP_2$)-specific phospholipase C (PLC) in PMT action on cultured fibroblasts and osteoblasts (Staddon *et al*., 1990; Staddon *et al*., 1991a; Lacerda *et al*., 1996; Mullan and Lax, 1998), which results in activation of the $IP_3$ pathway and causes $Ca^{2+}$ mobilization and PKC-dependent phosphorylation (Berridge, 1993).

Voltage-clamped *Xenopus* oocytes were used as a model system to demonstrate direct PMT-mediated stimulation of PLC activity and subsequent $IP_3$-induced intracellular $Ca^{2+}$ mobilization by monitoring the endogenous $Ca^{2+}$-dependent $Cl^-$ current evoked upon microinjection with PMT (Wilson *et al*., 1997). In addition, PMT activation of the PLC-$IP_3$ pathway in this system was found to be transient, but irreversible. Repeated microinjection of PMT did not elicit additional responses after the first injection, although subsequent injection with $IP_3$ still gave a response, supporting that the target of PMT action is upstream of $IP_3$ release (Wilson *et al*., 1997).

#### *Involvement of $G_q$-protein in PMT-induced PLC activation*

Receptor regulation of the $IP_3$ pathway is mediated either through protein tyrosine phosphorylation of PLCγ or G-protein activation of PLC β-isoforms. There are at least two G-protein-regulated $IP_3$ pathways that can be distinguished by their sensitivity to ADP-ribosylation by pertussis toxin (PTx) (Berstein *et al*., 1992, Camps *et al*., 1992, Wu *et al*., 1993). The βγ subunits of PTx-sensitive $G_{o/i}$-proteins preferentially stimulate PLCβ3 > PLCβ2 > PLCβ1. The α subunits of the PTx-insensitive $G_q$ family, including $G\alpha_q$ and $G\alpha_{11}$, stimulate PLCβ1 ≥ PLCβ3 » PLCβ2. Since PMT action results in the activation of a cellular $PIP_2$-specific PLC, it was possible that PMT-induced activation of the PLC-mediated $IP_3$ pathway could occur through one of these pathways. In keeping with this, PMT was found to facilitate PLC activation by G-protein-coupled receptor ligands such as bombesin, vasopressin, and endothelin (Murphy and Rozengurt, 1992).

To identify the intracellular signaling pathway involved in PMT-induced $IP_3$ signaling, specific antibodies against $G\alpha_{pan}$, $G\alpha_{q/11}$, $G\alpha_{i/o/t/z}$, $G\alpha_{s/olf}$, $G\beta_{pan}$, PLCβ1, PLCβ2, PLCβ3, or PLCγ1 were tested for their ability to block the PMT-induced $Cl^-$ currents in the oocyte system (Wilson *et al*., 1997). Only antibodies directed against PLCβ1, $G\alpha_{pan}$, or the C-terminus of $G\alpha_{q/11}$ blocked the PMT-mediated response in oocytes. GDPγS, a known inhibitor of Gα subunit-mediated signaling pathways, likewise blocked the PMT-induced response. The specific role of $G\alpha_q$ was further confirmed by over- and underexpression of mouse $G\alpha_q$ in *Xenopus* oocytes (Wilson *et al*., 1997). Overexpression of $G\alpha_q$ increased the PMT-induced response, whereas treatment with anti-sense $G\alpha_q$ RNA inhibited the response. These results established the direct involvement of $G\alpha_q$ protein in PMT-activation of PLCβ1. Studies using mouse knockout cell lines confirmed that PMT-induced PLC activation was exclusively dependent on $G\alpha_q$, and not other $G_q$ family members, such as $G_{11}$, $G_{12}$, or $G_{13}$ (other $G_q$ family members, $G_{14}$ or $G_{15/16}$, were not examined) (Zywietz *et al*., 2001).

Antibodies against Gβ subunit did not block but rather enhanced the PMT-induced response (Wilson *et al*., 1997), substantiating that Gβγ subunit activation of PLCβ2 by a $G_{i/o}$-dependent pathway was not involved in PMT action, and supporting that the anti-Gβ antibodies caused the dissociation of $G\alpha_q$ subunit from the heterotrimeric Gαβγ complex, such that $G\alpha_q$ could then be acted upon by PMT. The PMT-induced response was likewise enhanced by the release of $G\alpha_q$ subunit through sequestration of Gβγ subunits by PTx treatment. From these results, it was concluded that the monomeric $G\alpha_q$ subunit is the preferred target of PMT action on the PLC pathway. Based on these results, a model was proposed for PMT intracellular action on $G_q$-PLC-$IP_3$ signaling (see Figure 22.2).

#### *PMT interaction with $G_q$-protein*

Using a series of chimeras between $G\alpha_q$ and the closely related $G\alpha_{11}$ in $G\alpha_{q/11}$-knockout cells, a region of the helical domain was identified that is important for PMT-induced PLC activation (Orth *et al*., 2004). Within

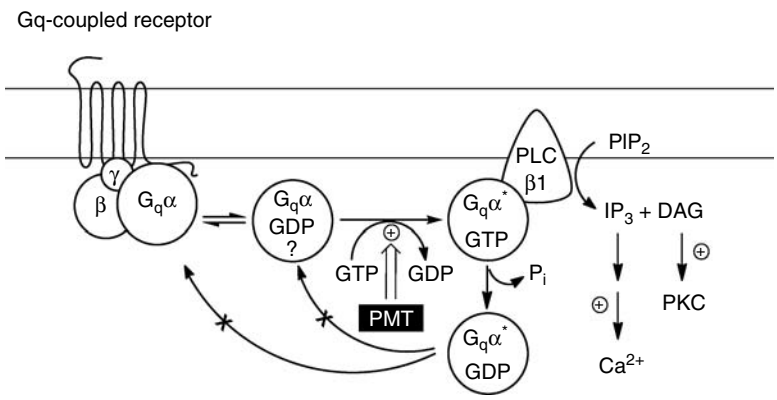



**FIGURE 22.2** Proposed model for PMT action on $G_q$-PLC signaling. Shown is a schematic representation of a proposed mechanism for PMT intracellular action, in which PMT acts on free GDP-bound $G\alpha_q$ and converts $G\alpha_q$ into an active form, presumably GTP-bound, which in turn stimulates PLCβ1. The activated PLCβ1 causes $PIP_2$ hydrolysis, leading to release of $IP_3$ and DAG and subsequent $Ca^{2+}$ mobilization, PKC activation, and downstream signaling. The PMT-induced response is transient due to the still-intact GTPase activity of $G\alpha_q$, which is stimulated by interaction with PLCβ1. The presumably modified $G\alpha_q$ can no longer be acted upon by PMT or be reassociated with the receptor-Gβγ complex.

the helical domain, exchange of Glu-105 or Asn-109 of $G\alpha_{11}$ with the corresponding His residues of $G\alpha_q$ resulted in a mutant $G\alpha_{11}$ protein that was able to mediate PMT-induced activation of PLC. However, the reciprocal exchange of the His residues in $G\alpha_q$ with the corresponding $G\alpha_{11}$ residues did not prevent PMT activation of PLC, suggesting that the differential activation of PLC mediated by these two proteins in response to PMT may be more complicated than a simple difference in recognition of the proteins by PMT.

Tyrosine phosphorylation of $G_{q/11}$ has been reported to regulate activation of $G_{q/11}$ signaling (Umemori *et al*., 1997; Umemori *et al*., 1999). Consistent with this, PMT appears to stimulate tyrosine phosphorylation of $G\alpha_q$; however, a mutant of PMT that was previously reported not to activate $G_q$ was also found to cause tyrosine phosphorylation of $G\alpha_q$ (Baldwin *et al*., 2003). Thus, the role of tyrosine phosphorylation in PMT action on $G_q$ is not clear.

### *PMT as a tool for studying $G_q$-PLC signaling*

PMT has been used as a selective activator of $G_q$-PLC signaling to demonstrate that $G_q$-coupled adrenergic receptor signaling in cardiomyocytes can differ between closely related animal species, such as mice and rats. For example, PMT was used to show that the $G_q$-PLC signaling pathway was still present and functional in mouse cardiomyocytes, even though α1-AR and endothelin receptors, which are known to selectively couple to PLC through $G_q$ in rat cardiomyocytes, were not functional as $G_q$-PLC regulators in the mouse cardiomyocytes (Sabri *et al*., 2000).

In neonatal rat cardiomyocytes, noradrenaline-stimulated $\alpha_{1A}$AR receptors were found to couple specifically to $G_{q/11}$ and not $G_{12/13}$, since overexpression of $G_{q/11}$-specific RGS4, but not $G_{12/13}$-specific Lsc-RGS, blocked $\alpha_{1A}$AR activation of PLCβ, which subsequently activated PLD through $Ca^{2+}$-independent PKC isoforms δ and ε (Gosau *et al*., 2002). PMT was used in this study to demonstrate the importance of $G_q$ and not $G_{11}$ in $\alpha_{1A}$AR activation of both PLC and downstream PLD.

PMT was used to show that histamine-induced catecholamine secretion from bovine adrenal chromaffin cells occurs through a PLC-independent membrane depolarization (Donald *et al*., 2002). In this study, PMT treatment caused an additive increase in basal and histamine-stimulated inositol phosphate levels, but not an increase or inhibition of adrenaline or noradrenaline secretion. The results obtained with PMT were consistent with other data, which showed that catecholamine secretion was not blocked by the PLC inhibitor ET-18-OCH3, by thapsigargin-depletion of intracellular $Ca^{2+}$ stores, or by $IP_3$-receptor inhibitors such as 2-aminoethoxydephenylborate or ryanodine plus caffeine (Donald *et al*., 2002).

Okada *et al*. used PMT to discriminate between $G_q$-dependent and $G_q$-independent $IP_3$ signaling induced by saccharin in frog rod taste cells (Okada *et al*., 2001). PMT treatment did not induce a response in the frog taste cells, suggesting the involvement of a Gβγ-coupled PLCβ2 isoform rather than a $G\alpha_q$-coupled PLCβ1 or PLCβ3 isoform in saccharin taste transduction.

PMT was recently used to demonstrate the involvement of $G_q$-PLC and $Ca^{2+}$ signaling in dendritic cell (DC) maturation (Bagley *et al*., 2004). In this study, the involvement of $Ca^{2+}$ release in PMT-stimulation of DC maturation was demonstrated by using xestospongin, an inhibitor that blocks $Ca^{2+}$ release from $IP_3$-gated intracellular stores.

PMT has also been used to study the role of $Ca^{2+}$ release in the cholinergic regulation of the circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) of the rat brain. Muscarinic regulation of the SCN clock occurs through $G_q$-coupled M1 mAChR, with sensitivity exhibited only during the night phase of the 24-hour clock cycle (Gillette *et al*., 2001). PMT treatment advanced the clock phase (i.e., caused the

clock to reset) in the same direction as activation of muscarinic receptors, such as carbachol-stimulated M1 mAChR, and $IP_3$-mediated $Ca^{2+}$ release in early night (L. Artinian, W. Yu, B. A. Wilson, E. Gratton, M. U. Gillette, unpublished data). Inhibitors of $IP_3$-mediated $Ca^{2+}$ release, such as xestospongin, blocked the PMT-induced phase shift.

### *PMT as a tool for studying GIRK signaling*

Since the initial observation that prolonged treatment with PMT uncouples $G_q$-PLC signaling such that no further activation occurs through $G_q$ (Wilson *et al*., 1997; Wilson *et al*., 2000), several investigators have demonstrated the effectiveness of using prolonged PMT treatment to down-regulate $G_q$-mediated inhibition of G-protein-coupled inward rectifying $K^+$ (GIRK) channels. Bunemann *et al*. and Lei *et al*. used prolonged PMT treatment to shut down $G_q$-mediated inhibition of GIRK channels in human embryonic kidney HEK-293 cells (Bunemann *et al*., 2000, Lei *et al*., 2001). They were able to demonstrate the existence of two different regulatory pathways for PTx-insensitive inhibition of GIRK channels: one involving PMT-insensitive $G_{i/o}$-coupled $M_2$ mAChR muscarinic or 5-$HT_{1A}$ serotonin receptors and the other involving PMT-sensitive $G_q$-coupled $\alpha_{1A}$AR or thyrotropin-releasing hormone (TRH) receptors. Meyer *et al*. further showed that PMT is superior to the commonly used aminosteroid PLC inhibitor U73122 for down-regulating $G_q$-mediated inhibition of GIRK channels in cardiomyocytes (Meyer *et al*., 2001).

## PMT effects on Rho signal transduction and cytoskeletal function

### *PMT and Rho signaling*

PMT has been shown to indirectly induce Rho-dependent tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and paxillin, which results in complex formation of $p125^{FAK}$ with the Src tyrosine kinase, actin stress fiber formation, and focal adhesion assembly (Lacerda *et al*., 1996; Ohnishi *et al*., 1998; Thomas *et al*., 2001). How PMT-mediated activation of the PLC-$IP_3$ signaling pathway promotes cytoskeletal rearrangement is not clear. However, tyrosine phosphorylation of $p125^{FAK}$ and paxillin is dependent on Rho, but appears to be independent of PKC activation or $Ca^{2+}$ mobilization (Lacerda *et al*., 1996; Ohnishi *et al*., 1998), two events resulting from PMT action on the PLC pathway. Inhibitors of serine/threonine protein kinases of the p160/ROK (Rho-associated coiled-coil-forming protein kinase) family, which are downstream targets of GTP-bound Rho, blocked PMT-induced phosphorylation of $p125^{FAK}$ (Thomas *et al*., 2001).

### *PMT and endothelial permeability*

PMT-induced Rho activation results in cell retraction and increased endothelial permeability (Essler *et al*., 1998). As shown in Figure 22.3, PMT disruption of endothelial integrity involves Rho-dependent activation of Rho kinase (ROK$\alpha$), which then inactivates myosin light chain (MLC) phosphatase PP1 and increases MLC phosphorylation and actin reorganization. This PMT-induced actin rearrangement was blocked by microinjection of the Rho inhibitor clostridial C3 transferase or by microinjection of the Rho-binding domain (RBD) or pleckstrin homology (PH) domain of ROK$\alpha$, which inhibits ROK$\alpha$ interaction with its regulators (Essler *et al*., 1998). PMT-mediated Rho activation has been speculated to be responsible for the observed vascular effects of PMT in dermonecrotic lesions from bite wounds (Aepfelbacher and Essler, 2001).

### *PMT and $G_q$-independent signaling*

PMT was shown to associate with vimentin (Shime *et al*., 2002), a component of intermediate filaments in cells, suggesting that this interaction may be involved in promoting cytoskeletal rearrangements. Another possibility is that PMT acts on the actin cytoskeleton indirectly through its action on Rho via $G_q$ or another yet unidentified $G_q$ family member (Katoh *et al*., 1998; Chikumi *et al*., 2002; Dutt *et al*., 2002; Vogt *et al*., 2003). In $G\alpha_q$- or $G\alpha_q/G\alpha_{11}$-deficient cells, PMT could still stimulate other cellular effects independent of $G_q$ or $G_{11}$, including Rho activation, Rho-dependent actin rearrangements and focal adhesions, and JNK and Erk mitogenic signaling (Zywietz *et al*., 2001). $G\alpha_{12/13}$ interaction with Rho-specific guanine nucleotide exchange factors can induce Rho-dependent responses (Sah *et al*., 2000). It may be that in the absence of $G_q$ or $G_{11}$, PMT acts on other related G proteins such as $G_{12}$ or $G_{13}$, both of which are known to activate Rho (Hart *et al*., 1998; Kozasa *et al*., 1998; Fukuhara *et al*., 1999; Gratacap *et al*., 2001; Kurose, 2003).

## PMT effects on mitogenic signal transduction

### *PMT and mitogenic signaling*

PMT acts on different cell types to initiate intracellular signal transduction events that result in DNA synthesis. PMT activates mature osteoclasts and induces proliferation and differentiation of periosteal (fibroblastic, osteogenic, and adipogenic) cells (Rozengurt *et al*., 1990; Mullan and Lax, 1996; Mullan and Lax, 1998). PMT stimulates the differentiation of preosteoclasts into osteoclasts and promotes osteoclast proliferation leading to bone resorption, while apparently inhibiting

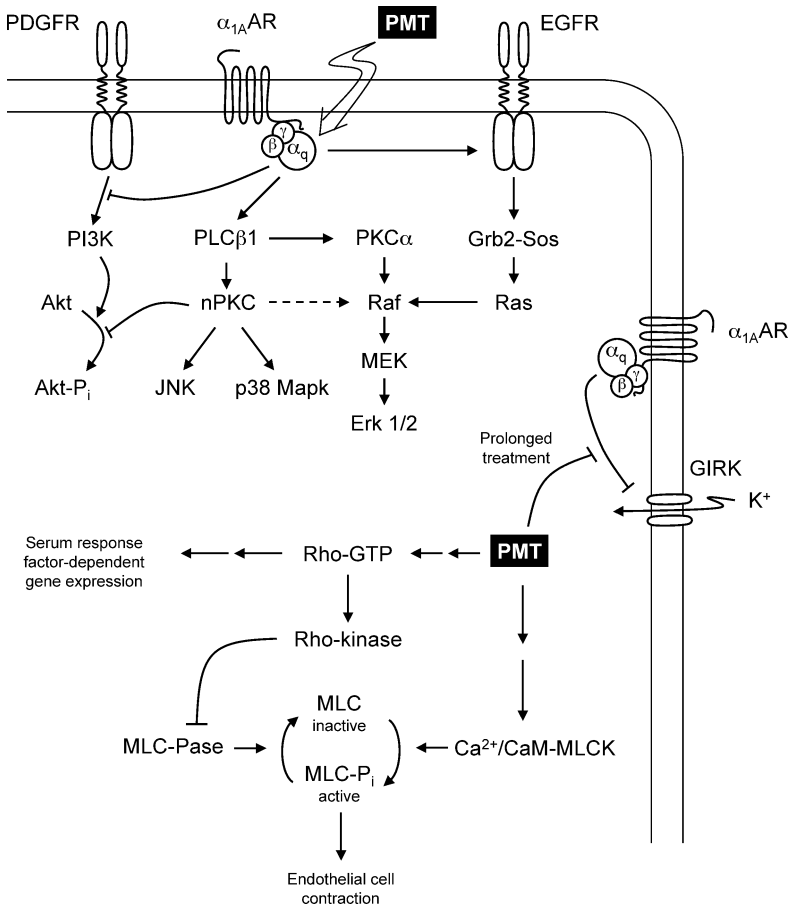



**FIGURE 22.3** Proposed model for PMT action on downstream signaling pathways. Shown is a schematic representation summarizing the proposed signaling pathways involved in PMT action. In this model activation of $G_q$ protein by PMT or $G_q$-coupled receptors, such as $\alpha_{1A}$AR, results in activation of PLCβ1, as well as transactivation of the EGF receptor via $G\alpha_q$ and inhibition of the PDGF receptor. In HEK-293 cells and cardiac fibroblasts, subsequent activation of the Erk1/2 kinase cascade is mediated by $G\alpha_q$ transactivation of the Ras-dependent EGF receptor tyrosine kinase pathway, with no significant contribution from the PLC-PKC pathway. In cardiomyocytes, PMT stimulates both the PLC-dependent $Ca^{2+}$ pathway and PKCα and novel nPKCs (PKCδ and PKCε), which in turn leads to activation of Erk1/2, as well as p38MAPK and JNK cascades. PMT both inhibits PDGF receptor-dependent PI 3-kinase (PI3K) activation and prevents subsequent activation of the Akt-dependent survival pathway. Release of $Ca^{2+}$ leads to activation of $Ca^{2+}$/calmodulin-dependent myosin light chain kinase ($Ca^{2+}$/CaM-MLCK), which then phosphorylates and activates MLC. PMT also stimulates the activation of Rho GTPase, stimulating Rho kinase to phosphorylate and thereby inactivates MLC phosphatase (MLC-Pase), which in turn prevents dephosphorylation of MLC, keeping it in its active state and causing cell contraction and endothelial permeability. Prolonged treatment with PMT down-regulates $G\alpha_q$-mediated signaling, including $G\alpha_q$-dependent inhibition of GIRK channels.

bone regeneration by osteoblasts (Sterner-Kock *et al.*, 1995; Mullan and Lax, 1998). In cultured epithelial cells, such as calf and monkey (Vero) kidney cells, calf testis, and bovine embryonic lung cells, PMT appears to cause morphological changes and cytopathic effects (Pennings and Storm, 1984; Rutter and Luther, 1984; Pettit *et al.*, 1993a; Sterner-Kock *et al.*, 1995; Dudet *et al.*, 1996). In cultured mesenchymal cells, such as murine, rat, and human fibroblasts, PMT action is mitogenic and initiates DNA synthesis and cell division (Rozengurt *et al.*, 1990; Mullan and Lax, 1996), which can promote anchorage-independent cell growth in soft agar (Higgins *et al.*, 1992), leading to the speculation that it can promote cell transformation and even cancer (Lax and Thomas, 2002). In agreement with these findings, flow cytometry analysis revealed that PMT stimulates fibroblast cells to move from the $G_1$ phase into and through the S phase without triggering apoptosis (Wilson *et al.*, 2000).

While PMT induced confluent Swiss 3T3 cells to increase in cell number up to three-fold, forming dense monolayers over the course of 4–6 days, cell cycle analysis revealed, however, that after the initial mitogenic response to PMT, cells subsequently arrested primarily in $G_1$ and became unresponsive to further PMT treatment (Wilson *et al.*, 2000). Consistent with these findings, PMT initially up-regulated the expression of a number of cell cycle markers, including the protooncogene c-Myc; the cyclins D1, D2, D3, and E; p21; PCNA; and the Rb proteins p107 and p130, yet continued expression of these markers, and hence continued proliferation, was not sustained (Wilson *et al.*, 2000). On the other hand, PMT exhibited a differential effect on epithelial-like cells. In confluent Vero cells, PMT induced rapid, dramatic morphological changes, but there was no increase in cell numbers or in the rate of DNA synthesis. Furthermore, PMT failed to up-regulate PCNA or cyclins D3 and E, markers critical for driving cells from $G_1$ into S phase; and hence, little or no cell cycle progression occurred in confluent Vero cells, which was further substantiated by flow cytometry analysis (Wilson *et al.*, 2000).

Mitogenic signaling stimulated by PMT appears to be different for different cell types. In some cells, PMT stimulation of Erk signaling occurs via $G_q$-dependent transactivation of epidermal growth factor (EGF)

receptor in a Ras-dependent manner, whereas in other cells, it occurs via a PKC-dependent, Ras-independent pathway (see Figure 22.3). In HEK-293 cells, both PMT and endogenous $G_q$-coupled α-thrombin receptors induced Ras-dependent Erk activation via PKC-independent transactivation of the EGF receptor (Seo *et al*., 2000). Expression of a dominant-negative mutant of the G-protein-coupled receptor kinase (GRK2) or a C-terminal peptide of $G\alpha_q$ ($G\alpha_{q305\text{-}359}$) blocked Erk activation by PMT and α-thrombin in these cells. In addition, PMT-stimulated Erk activation was blocked by dominant-negative inhibitors of mSos1 and Ha-Ras, as well as by an EGF-receptor-specific inhibitor tyrphostin (AG1478), but not by a PKC-specific inhibitor (GF109203X). PMT was similarly found to stimulate Erk activation via EGF receptor transactivation in cardiac fibroblasts; however, in cardiomyocytes novel PKC isoforms mediated PMT activation of Erk, p38MAPK, and JNK independently of the EGF receptor (Sabri *et al*., 2002).

PMT-mediated transactivation of tyrosine kinase signaling via $G_q$ activation was also reported for the generation of inositol phosphoglycans, which are second messengers of insulin signaling (Sleight *et al*., 2002). In this study, PMT stimulated the production of inositol phosphoglycans and interaction between the $G_q$ signaling pathway and the insulin receptor tyrosine kinase pathway, as evidenced by immunogold-labeling showing co-localization of the insulin receptor β subunit (IRβ) and $G\alpha_{q/11}$ in partially purified rat liver membranes, enriched in PLCβ1, clathrin, and caveolin-1.

### *PMT and apoptosis*

In neonatal rat cardiomyocytes, PMT and norepinephrine similarly induced cardiac hypertrophy, as evidenced by cellular enlargement of the cardiomyocytes, enhanced sarcomeric organization, and increased atrial natriuretic factor expression (Sabri *et al*., 2002). In these cells, PMT activated Erk, p38MAPK, and JNK via activation of PLC and novel PKC isoforms. PMT also decreased basal Akt activation by preventing Akt phosphorylation through the activation of EGF or insulin-like growth factor-1 (IGF-1), which led to cardiomyocyte susceptibility to apoptotic agents such as $H_2O_2$. This effect of PMT is reminiscent of what occurs upon intense, prolonged stimulation of cardiomyocytes by $G_q$-coupled receptors (Adams *et al*., 1998), where $G_q$-coupled receptor (or PMT) activation of $G_q$ stimulates cardiac hypertrophy, while inhibiting the Akt survival pathway and thereby promoting cardiomyocyte susceptibility to apoptosis. It is not known if PMT might cause similar effects in other cells, such as osteoblasts.

## PMT STRUCTURE AND FUNCTIONAL ORGANIZATION

### Sequence analysis

When the *toxA* gene was first isolated, cloned, and sequenced, no significant homologies to the primary sequences of any other proteins were found (Buys *et al*., 1990; Lax and Chanter, 1990; Lax *et al*., 1990; Petersen, 1990). However, shortly thereafter, additional members of the PMT dermonecrotic toxin family were reported, including the cytotoxic necrotizing factors type 1 and 2 (CNF1 and CNF2) from pathogenic *E. coli* (Falbo *et al*., 1993; Oswald *et al*., 1994), the dermonecrotic toxin (DNT) from *Bordetella* species (Walker and Weiss, 1994; Pullinger *et al*., 1996), and more recently the CNF homologue from *Yersinia pseudotuberculosis* ($CNF_Y$) (Lockman *et al*., 2002). CNF1 and CNF2 share 85% sequence identity (Oswald *et al*., 1994), and $CNF_Y$ shares ~65% homology with the *E. coli* CNFs (Lockman *et al*., 2002). PMT and CNF1/2 share 46% sequence similarity in their N-terminal 500 amino acids (Falbo *et al*., 1993; Oswald *et al*., 1994). Limited sequence similarity (<25%) between PMT and DNT (Walker and Weiss, 1994; Pullinger *et al*., 1996) can also be found in their N-terminal regions (Wilson *et al*., 1999). In addition, DNT and the CNFs share 55% similarity in their C-terminal 300 amino acids (see Figure 22.4).

### Localization of functional domains

Because PMT has cell binding, membrane translocation, and intracellular activities, it is believed to be composed of multiple functional domains and to belong to the family of AB toxins, as exemplified by diphtheria toxin. When the sequences of PMT and the related CNFs and DNT were first reported, there were no other matches in the databases that could provide clues about the functional domain structure of these proteins, although limited similarities in the C-terminus to putative metal-binding sites (Petersen, 1990), a

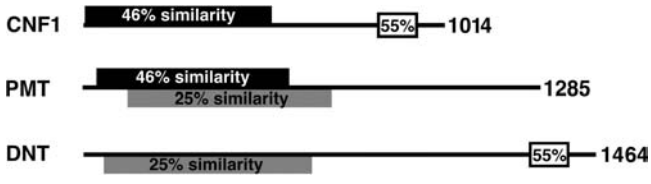



**FIGURE 22.4** Sequence comparison of the dermonecrotic toxins. Shown is a schematic representation indicating the regions of sequence similarity between PMT, CNF1, and DNT.

hydrophobic region between residues 380–470 (Lax *et al*., 1990), two amphipathic α-helices (Petersen, 1990), and a putative ADP-ribosyltransferase active-site His-Glu-Trp motif in the N-terminus (Lax *et al*., 1990) were described.

Single and double Ala substitution of the His and Glu residues of the putative ADP-ribosylation motif in PMT (His-29, Glu-155, and Trp-160) failed to diminish biological activity of the mutant proteins compared to wild-type PMT (Ward *et al*., 1994). Another study designed to detect toxin-catalyzed ADP-ribosylation in intact cells, in which the cellular pool of $NAD^+$ was metabolically labeled with [2-$^3$H] adenine, likewise failed to demonstrate PMT-mediated ADP-ribosylation of cellular proteins (Staddon *et al*., 1991b).

In a study to identify nontoxic, immunogenic vaccine candidates of PMT, four deletion mutants were expressed in *E. coli,* and the purified recombinant proteins were tested for biological activity and protective immunity (Petersen *et al*., 1991). Deletion of residues 175–247 generated an unstable recombinant protein that was found primarily in inclusion bodies. Deletion of residues 507–567 yielded a recombinant protein with 0.2% wild-type biological activity in the EBL cell assay and with 2% wild-type activity in dermonecrotic lesion and mouse lethality assays. Deletion of residues 28–149 resulted in a non-toxic protein that was able to provide protective immunity, while deletion of residues 1130–1285 resulted in a non-toxic protein that was unable to provide protective immunity. These results suggested that the C-terminus contains important immunological determinants required for neutralization of toxin and for providing protective immunity.

Specific antibodies against peptides derived from the N-terminus ($ToxA_{28\text{-}42}$), but not the C-terminus ($ToxA_{1239\text{-}1253}$), of PMT blocked the PMT-induced $Ca^{2+}$-dependent $Cl^-$ current in *Xenopus* oocytes (Wilson 1997). Consistent with this, microinjection of purified recombinant N-terminal (1–568, 1–505), but not C-terminal (505–1285, 1059–1285), deletion fragments of PMT into oocytes elicited toxin-induced responses (Wilson *et al*., 1999). However, other studies suggest that it is the C-terminus of PMT that has intracellular activity and that the N-terminus is the receptor-binding domain. In one of these studies, electroporation was used to introduce PMT fragments directly into EBL cells, and results indicated that C-terminal (581–1285), but not N-terminal (1–483, 1–1130), deletion fragments elicited morphology changes similar to intact PMT (Busch *et al*., 2001). In another study, fusion proteins of N-terminal (1–506) and C-terminal (681–1285, 849–1285) fragments of PMT with GST affinity tags at the N-terminus were microinjected into Swiss 3T3 cells and compared to intact PMT for morphology changes and biological activity (Pullinger *et al*., 2001). Results from this study suggested that the GST-fusion with residues 681–1285 induced morphology changes and mitogenesis similar to intact PMT, while the GST-fusion with the N-terminal fragment did not. The reason for the discrepancy between the results from these studies is not yet clear, but may be due to the nature of the properties of the recombinant PMT fragments and fusion proteins under the different experimental conditions used or may reflect that PMT has multiple binding and/or activity determinants.

PMT contains eight Cys residues at positions 26, 113, 230, 257, 793, 905, 1159, and 1165. Mutation of each of these residues revealed that there are probably no essential disulfide bonds and that only mutation of Cys-1159 and Cys-1165 resulted in reduced biological activity of PMT (Ward *et al*., 1998), with the Cys-1159-Gly mutant being about 10-fold reduced in mitogenic activity and the Cys-1165-Gly and Cys-1165-Ser mutants being greater than 10,000-fold reduced in activity. Although the role of the Cys-1165 residue in PMT activity was not determined in this study, another study in which wild-type and mutant proteins were electroporated directly into cells revealed that the Cys-1165-Ser mutant was unable to cause an increase in inositol phosphate levels (Busch *et al*., 2001).

Pretreatment of PMT with diethyl pyrocarbonate (DEPC), which covalently modifies His residues, inhibited the biological activity of PMT (Orth *et al*., 2003). DEPC-treated C-terminal fragment (581–1285) delivered by electroporation into EBL cells was blocked in its ability to stimulate PLC activity, as evidenced by reduced inositol phosphate accumulation. Consequently, the eight His residues located in the C-terminus of PMT (at positions 622, 635, 804, 1001, 1202, 1205, 1223, and 1228) were mutated to Ala or Leu, and their biological activities were compared with intact PMT (Orth *et al*., 2003). All but three of the mutants were fully active. The mutants His-1205-Leu and His-1223-Leu, and to a lesser extent His-1228-Leu, were significantly reduced in activity, suggesting the involvement of these His residues in PMT biological activity. In keeping with the earlier suggestion that this region contains a putative metal-binding motif (Petersen, 1990), it was suggested that these His residues might be involved in metal binding (Orth *et al*., 2003).

Recently, the involvement of the two predicted hydrophobic helices (residues 402–423 and 437–457) and the connecting hydrophilic loop (residues 424–436) in membrane translocation of PMT was tested by substitution of the acidic residues in the loop (Asp-425, Asp-431, Glu-434) with Lys (Baldwin *et al.,* 2004). Individual mutation of each of these residues caused up to a six-fold reduction in toxin translocation into

cells after binding to the surface in the presence of endocytosis inhibitor and then pulsing with low pH. On the other hand, mutation of Asp-401, which is outside of the predicted helix-loop-helix motif, completely abolished PMT activity. The reason for this observation was not determined, and it is not clear how this fits into the proposed model for translocation. Interestingly, comparison of the results for mutation of these residues in PMT with the results for the corresponding residues in CNF1 (Pei *et al*., 2001) suggested that if these regions are involved in membrane translocation then the mechanisms of translocation differ between PMT and CNF1 (Baldwin *et al*., 2004).

## Comparison with related toxins

### *Sequence comparison with DNT and CNF*

For DNT and the CNFs, the intracellular activity occurs through constitutive activation of the small G-protein RhoA (Oswald *et al*., 1994; Fiorentini *et al*., 1997; Flatau *et al*., 1997; Horiguchi *et al*., 1997; Schmidt *et al*., 1997), which regulates actin cytoskeletal function and is responsible for the formation of actin stress fibers and other cytoskeletal rearrangements. This activity is localized to the C-terminal 300 residues of DNT and the CNFs (Lemichez *et al*., 1997). However, PMT does not share this C-terminal sequence similarity. PMT also does not deamidate or polyaminate RhoA (Ohnishi *et al*., 1998) and does not act directly on RhoA (Lacerda *et al*., 1997; Ohnishi *et al*., 1998; Horiguchi, 2001). Thus, it is unlikely that PMT has a deamidase domain similar to that found in CNF or DNT.

Both CNFs are proteins of 1014 amino acid residues that share 85% sequence identity and 99% similarity (Falbo *et al*., 1993; Oswald *et al*., 1994). Because of the high homology between the two CNFs, it is believed that CNF2 has similar intracellular properties to that of CNF1. CNF1 has been shown to deamidate Gln-63 of RhoA (Flatau *et al*., 1997; Schmidt *et al*., 1997), a residue essential for the GTPase activity. The RhoA-deamidase domain also possesses transglutaminase activity and can incorporate alkylamines into RhoA at the Gln-63 position (Schmidt *et al*., 2001). This activity involves two essential C-terminal residues, Cys-866 and His-881 (Schmidt *et al*., 1998). The crystal structure of the Rho-activating domain of CNF1 is available (Buetow *et al*., 2001). Recent evidence suggests that the receptor-binding domain is located at the N-terminus (Fabbri *et al*., 1999; Meysick *et al*., 2001; Pei *et al*., 2001).

DNT is a 1464 amino acid protein produced by various *Bordetella* species (Walker and Weiss, 1994). The DNTs produced by *B. bronchiseptica, B. pertussis*, and *B. parapertussis* are nearly identical (>99% homology). DNT causes dermonecrosis in mice, forming hemorrhagic lesions at the site of inoculation. In osteoblast-like MC3T3-E1 cells, DNT changes cell morphology and inhibits differentiation into osteoblasts (Horiguchi *et al*., 1993). In fibroblast cells, DNT, like the CNFs, stimulates actin stress fiber formation and cytoskeletal rearrangements via Gln-63 deamidation and constitutive activation of RhoA (Horiguchi *et al*., 1995; Horiguchi *et al*., 1997; Ohnishi *et al*., 1998). DNT has also been shown to modify the Rho-related GTPases Rac1 and Cdc42 (Horiguchi *et al*., 1997; Masuda *et al*., 2002). Evidence suggests that the receptor-binding domain is located at the N-terminus (Matsuzawa *et al*., 2002).

### *Activity comparison with DNT and CNF*

One question of interest is the relationship between the structural similarities and the activities of the three toxins. PMT, DNT, and the CNFs cause dermonecrotic lesions at the site of injection in animal skin assays. How the molecular activity of these toxins translates into dermonecrotic lesions is not clear. Considering that PMT has been shown to induce IL-6 production in certain cells (Rosendal *et al*., 1995), it is possible that an inflammatory response contributes to dermonecrosis. On the cellular level, they all appear to cause similar, but not identical, effects on the cytoskeleton in cultured cells (Dudet *et al*., 1996; Lacerda *et al*., 1996; Horiguchi *et al*., 1997; Lacerda *et al*., 1997; Ohnishi *et al*., 1998). All three toxins stimulate DNA synthesis and actin cytoskeletal rearrangements, although through different mechanisms (Ohnishi *et al*., 1998).

CNF1 induces macropinocytosis in epithelial cells (Fiorentini *et al*., 2001) and phagocytic behavior in HEp-2 cells through activation of membrane ruffling (Falzano *et al*., 1993), which can trigger entry of noninvasive bacteria into cells and may contribute to its role in pathogenicity. Unlike PMT, the CNFs and DNT do not induce PLC activation, PKC activation, or $Ca^{2+}$ mobilization (Ohnishi *et al*., 1998). DNT and the CNFs stimulate DNA synthesis, but cell division does not occur; instead, cytokinesis is blocked during mitosis, resulting in large bi- and multinuclear cells. On the other hand, PMT stimulates DNA synthesis, but without adversely affecting cell division, and instead causes cell proliferation. Whereas PMT was shown to activate the MAPK pathway (Seo *et al*., 2000; Wilson *et al*., 2000), CNF1 and DNT did not (Lacerda *et al*., 1997). All three toxins have been shown to induce cytoskeletal changes through Rho-dependent tyrosine phosphorylation of p125FAK and paxillin; however, CNF and DNT do so by direct activation of Rho, whereas PMT appears to do so through indirect activation of Rho (Horiguchi *et al*., 1995; Lacerda *et al*., 1996; Lacerda *et al*., 1997; Thomas *et al*., 2001).

## CONCLUSION

Compared to the current knowledge of the structure and function of other bacterial protein toxins, particularly for the ADP-ribosylating, α-hemolysin-like, anthrax, and neurotoxin families of toxins, our understanding of PMT structure and activity is at a much earlier stage, and a number of outstanding questions remain about its functional organization, additional intracellular targets, and biochemical activity. Yet, because of its unique action on $G_q$, PMT is already being used as a potent research tool in deciphering cellular signal transduction pathways involving $G_q$-proteins. PMT facilitation of $G_q\alpha$-protein coupling to PLCβ1 causes the same cellular responses elicited by $G_q$-protein-coupled receptors. Down-regulation of $G_q$-PLC signaling upon prolonged PMT treatment also allows for distinguishing between PTx-sensitive and PTx-insensitive signaling pathways that involve $G_q$. Understanding the biochemical mechanism of PMT action may thus afford unique insight into the molecular signaling events involved in regulation of cell function, growth, and differentiation.

## ACKNOWLEDGMENTS

Some of the work reported here was supported by grants from the National Institutes of Health (NIAID/AI38396) and from the United States Department of Agriculture (NRI/1999-02295) (to B.A.W.).

## REFERENCES

Ackermann, M.R., Register, K.B., Stabel, J.R., Gwaltney, S.M., Howe, T.S. and Rimler, R.B. (1996). Effect of *Pasteurella multocida* toxin on physeal growth in young pigs. *Am. J. Vet. Res*., **57,** 848–852.

Adams, J.W., Sakata, Y., Davis, M.G., Sah, V.P., Wang, Y., Liggett, S. B., Chien, K.R., Brown, J.H. and Dorn, G.W., 2nd (1998). Enhanced $G\alpha_q$ signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc. Natl. Acad. Sci. USA*, **95,** 10140–10145.

Adler, B., Bulach, D., Chung, J., Doughty, S., Hunt, M., Rajakumar, K., Serrano, M., van Zanden, A., Zhang, Y. and Ruffolo, C. (1999). Candidate vaccine antigens and genes in *Pasteurella multocida*. *J. Biotechnol*., **73,** 83–90.

Aepfelbacher, M. and Essler, M. (2001). Disturbance of endothelial barrier function by bacterial toxins and atherogenic mediators: a role for Rho/Rho kinase. *Cell Microbiol*., **3,** 649–658.

Andresen, L.O. (1989). Undersogelser over *Pasteurella multocida* DNA. Pavisning af et mobiliserbart DNA-element indeholdende PMT-genet i *P. multocida*'s kromosom. *Danish Acad. Techn. Scienc., Erhvervsforskerrapport, EF* **229.**

Bagley, K.C., Abdelwahab, S.F., Tuskan, R.G. and Lewis, G.K. (2004). Calcium signaling through phospholipase C activates dendritic cells to mature and is necessary for the activation and maturation of dendritic cells induced by diverse agonists. *Clin. Diagn. Lab. Immunol*., **11,** 77–82.

Baldwin, M.R., Lakey, J.H. and Lax, A.J. (2004). Identification and characterization of the *Pasteurella multocida* toxin translocation domain. *Mol. Microbiol*., **54,** 239–250.

Baldwin, M.R., Pullinger, G.D. and Lax, A.J. (2003). *Pasteurella multocida* toxin facilitates inositol phosphate formation by bombesin through tyrosine phosphorylation of G alpha q. *J. Biol. Chem*., **278,** 32719–32725.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signaling. *Nature*, **361,** 315–325.

Berstein, G., Blank, J.L., Smrka, A.V., Higashijima, T., Sternweis, P.C., Exton, J.H. and Ross, E.M. (1992). Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, $G_{q/11}$, and phospholipase Cβ-1. *J. Biol. Chem*., **267,** 8081–8088.

Bording, A. and Foged, N.T. (1991). Characterization of the immunogenicity of formaldehyde detoxified *Pasteurella multocida* toxin. *Vet. Microbiol*., **29,** 267–280.

Brockmeier, S.L., Palmer, M.V., Bolin, S.R. and Rimler, R.B. (2001). Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive, and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. *Am. J. Vet. Res*., **62,** 521–525.

Buetow, L., Flatau, G., Chiu, K., Boquet, P. and Ghosh, P. (2001). Structure of the Rho-activating domain of *Escherichia coli* cytotoxic necrotizing factor 1. *Nat. Struct. Biol*., **8,** 584–588.

Bunemann, M., Meyer, T., Pott, L. and Hosey, M. (2000). Novel inhibition of Gβγ-activated potassium currents induced by M(2) muscarinic receptors via a pertussis toxin-insensitive pathway. *J. Biol. Chem*., **275,** 12537–12545.

Busch, C., Orth, J., Djouder, N. and Aktories, K. (2001). Biological activity of a C-terminal fragment of *Pasteurella multocida* toxin. *Infect. Immun*., **69,** 3628–3634.

Buys, W.E., Smith, H.E., Kamps, A.M., Kamp, E.M. and Smits, M.A. (1990). Sequence of the dermonecrotic toxin of *Pasteurella multocida* ssp. *multocida. Nucleic Acids Res*., **18,** 2815–2816.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J. and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C-β2 by G protein βγ-subunits. *Nature*, **360,** 684–686.

Chanter, N. (1990). Advances in atrophic rhinitis and toxigenic *Pasteurella multocida* research. *Pig News Inform*., **11,** 503–506.

Chanter, N., Magyar, T. and Rutter, J.M. (1989). Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. *Res. Vet. Sci*., **47,** 48–53.

Chanter, N. and Rutter, J.M. (1990). Colonization by *Pasteurella multocida* in atrophic rhinitis of pigs and immunity to the osteolytic toxin. *Vet. Microbiol*., **25,** 253–265.

Chanter, N., Rutter, J.M. and Mackenzie, A. (1986). Partial purification of an osteolytic toxin from *Pasteurella multocida. J. Gen. Microbiol*., **132,** 1089–1097.

Cheville, N.F. and Rimler, R.B. (1989). A protein toxin from *Pasteurella multocida* type D causes acute and chronic hepatic toxicity in rats. *Vet. Pathol*., **26,** 148–157.

Cheville, N.F., Rimler, R.B. and Thurston, J.R. (1988). A toxin from *Pasteurella multocida* type D causes acute hepatic necrosis in pigs. *Vet. Pathol*., **25,** 518–520.

Chikumi, H., Vazquez-Prado, J., Servitja, J.M., Miyazaki, H. and Gutkind, J.S. (2002). Potent activation of RhoA by $G\alpha_q$ and $G_q$-coupled receptors. *J. Biol. Chem*., **277,** 27130–27134.

Chrisp, C.E. and Foged, N.T. (1991). Induction of pneumonia in rabbits by use of a purified protein toxin from *Pasteurella multocida. Am. J. Vet. Res*., **52,** 56–61.

Davies, R.L., MacCorquodale, R., Baillie, S. and Caffrey, B. (2003). Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *J. Med. Microbiol*., **52,** 59–67.

Davies, R.L., MacCorquodale, R. and Reilly, S. (2004). Characterization of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine, and porcine origin. *Vet. Microbiol.*, **99,** 145–158.

de Jong, M.F. and Akkermans, J.P. (1986). Investigation into the pathogenesis of atrophic rhinitis in pigs. I. Atrophic rhinitis caused by *Bordetella bronchiseptica* and *Pasteurella multocida* and the meaning of a thermolabile toxin of *P. multocida. Vet. Quarterly*, **8,** 204–214.

de Jong, M.F., de Wachter, J.C. and de Marel, G.M. (1986). Investigation into the pathogenesis of atrophic rhinitis in pigs. II. AR induction and protection after intramuscular injections cell-free filtrates and emulsions containing AR toxin of *Pasteurella multocida. Vet. Quarterly*, **8,** 215–224.

Deeb, B.J. and DiGiacomo, R.F. (2000). Respiratory diseases of rabbits. *Vet. Clinics North America. Exotic Animal Practice*, **3,** 465–480.

Deeb, B.J., DiGiacomo, R.F., Bernard, B. L. and Silbernagel, S. M. (1990). *Pasteurella multocida* and *Bordetella bronchiseptica* infections in rabbits. *J. Clin. Microbiol.*, **28,** 70–75.

DiGiacomo, R.F., Deeb, B. J., Brodie, S. J., Zimmerman, T. E., Veltkamp, E. R. and Chrisp, C. E. (1993). Toxin production by *Pasteurella multocida* isolated from rabbits with atrophic rhinitis. *Am. J. Vet. Res.*, **54,** 1280–1286.

DiGiacomo, R.F., Deeb, B. J., Giddens, W. E., Jr., Bernard, B. L. and Chengappa, M.M. (1989). Atrophic rhinitis in New Zealand white rabbits infected with *Pasteurella multocida. Am. J. Vet. Res.*, **50,** 1460–1465.

DiGiacomo, R.F., Garlinghouse, L.E., Jr. and Van Hoosier, G.L., Jr. (1983). Natural history of infection with *Pasteurella multocida* in rabbits. *J. Am. Vet. Med. Assoc.*, **183,** 1172–1175.

DiGiacomo, R.F., Taylor, F.G., Allen, V. and Hinton, M. H. (1990). Naturally acquired *Pasteurella multocida* infection in rabbits: immunological aspects. *Lab. Animal Sci.*, **40,** 289–292.

DiGiacomo, R.F., Xu, Y. M., Allen, V., Hinton, M. H. and Pearson, G. R. (1991). Naturally acquired *Pasteurella multocida* infection in rabbits: clinicopathological aspects. *Can. J. Vet. Res.*, **55,** 234–238.

Dominick, M.A. and Rimler, R.B. (1986). Turbinate atrophy in gnotobiotic pigs intranasally inoculated with protein toxin isolated from type D *Pasteurella multocida. Am. J. Vet. Res.*, **47,** 1532–1536.

Dominick, M.A. and Rimler, R.B. (1988). Turbinate osteoporosis in pigs following intranasal inoculation of purified *Pasteurella* toxin: histomorphometric and ultrastructural studies. *Vet. Pathol.*, **25,** 17–27.

Donald, A.N., Wallace, D.J., McKenzie, S. and Marley, P.D. (2002). Phospholipase C-mediated signaling is not required for histamine-induced catecholamine secretion from bovine chromaffin cells. *J. Neurochem.*, **81,** 1116–1129.

Dowling, A., Hodgson, J.C., Dagleish, M.P., Eckersall, P.D. and Sales, J. (2004). Pathophysiological and immune cell responses in calves prior to and following lung challenge with formalin-killed *Pasteurella multocida* biotype A:3 and protection studies involving subsequent homologous live challenge. *Vet. Immunol. Immunopathol.*, **100,** 197–207.

Dowling, A., Hodgson, J.C., Schock, A., Donachie, W., Eckersall, P.D. and McKendrick, I.J. (2002). Experimental induction of pneumonic pasteurellosis in calves by intratracheal infection with *Pasteurella multocida* biotype A:3. *Res. Vet. Sci.*, **73,** 37–44.

Dudet, L.I., Chailler, P., Dubreuil, J.D. and Martineau-Doize, B. (1996). *Pasteurella multocida* toxin stimulates mitogenesis and cytoskeleton reorganization in Swiss 3T3 fibroblasts. *J. Cell Physiol.*, **168,** 173–182.

Dutt, P., Kjoller, L., Giel, M., Hall, A. and Toksoz, D. (2002). Activated $G\alpha_q$ family members induce Rho GTPase activation and Rho-dependent actin filament assembly. *FEBS Lett.*, **531,** 565–569.

Elias, B., Boros, G., Albert, M., Tuboly, S., Gergely, P., Papp, L., Barna Vetro, I., Rafai, P. and Molnar, E. (1990). Clinical and pathological effects of the dermonecrotic toxin of *Bordetella bronchiseptica* and *Pasteurella multocida* in specific-pathogen-free piglets. *Nippon Juigaku Zasshi—Jap. J. Vet. Sci.*, **52,** 677–688.

Elias, B., Gergely, P., Boros, G. and Varro, R. (1986). Epizootiological studies on porcine atrophic rhinitis. X. Study of the heat-labile exotoxin (HLT) of *Pasteurella multocida* in mice. *Acta Veterinaria Hungarica*, **34,** 137–143.

Elling, F. and Pedersen, K.B. (1985). The pathogenesis of persistent turbinate atrophy induced by toxigenic *Pasteurella multocida* in pigs. *Vet. Pathol.*, **22,** 469–474.

Elling, F., Pedersen, K.B., Hogh, P. and Foged, N.T. (1988). Characterization of the dermal lesions induced by a purified protein from toxigenic *Pasteurella multocida. APMIS*, **96,** 50–55.

Essler, M., Hermann, K., Amano, M., Kaibuchi, K., Heesemann, J., Weber, P.C. and Aepfelbacher, M. (1998). *Pasteurella multocida* toxin increases endothelial permeability via Rho kinase and myosin light chain phosphatase. *J. Immunol.*, **161,** 5640–5646.

Fabbri, A., Gauthier, M. and Boquet, P. (1999). The 5′ region of *cnf1* harbors a translational regulatory mechanism for CNF1 synthesis and encodes the cell-binding domain of the toxin. *Mol. Microbiol.*, **33,** 108–118.

Falbo, V., Pace, T., Picci, L., Pizzi, E. and Caprioli, A. (1993). Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli. Infect. Immun.*, **61,** 4909–4914.

Falzano, L., Fiorentini, C., Donelli, G., Michel, E., Kocks, C., Cossart, P., Cabanie, L., Oswald, E. and Boquet, P. (1993). Induction of phagocytic behavior in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. *Mol. Microbiol.*, **9,** 1247–1254.

Felix, R., Fleisch, H. and Frandsen, P.L. (1992). Effect of *Pasteurella multocida* toxin on bone resorption *in vitro. Infect. Immun.*, **60,** 4984–4988.

Fiorentini, C., Fabbri, A., Flatau, G., Donelli, G., Matarrese, P., Lemichez, E., Falzano, L. and Boquet, P. (1997). *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1), a toxin that activates the Rho GTPase. *J. Biol. Chem.*, **272,** 19532–19537.

Fiorentini, C., Falzano, L., Fabbri, A., Stringaro, A., Logozzi, M., Travaglione, S., Contamin, S., Arancia, G., Malorni, W. and Fais, S. (2001). Activation of rho GTPases by cytotoxic necrotizing factor 1 induces macropinocytosis and scavenging activity in epithelial cells. *Mol. Biol. Cell*, **12,** 2061–2073.

Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature*, **387,** 729–733.

Foged, N.T. (1988). Quantitation and purification of the *Pasteurella multocida* toxin by using monoclonal antibodies. *Infect. Immun.*, **56,** 1901–1906.

Foged, N.T. (1991). Detection of stable epitopes on formaldehyde-detoxified *Pasteurella multocida* toxin by monoclonal antibodies. *Vaccine*, **9,** 817–824.

Foged, N.T. (1992). *Pasteurella multocida* toxin. The characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. *APMIS Suppl.*, **25,** 1–56.

Foged, N.T., Nielsen, J.P. and Jorsal, S.E. (1989). Protection against progressive atrophic rhinitis by vaccination with *Pasteurella multocida* toxin purified by monoclonal antibodies. *Vet. Record*, **125,** 7–11.

Foged, N.T., Pedersen, K.B. and Elling, F. (1987). Characterization and biological effects of the *Pasteurella multocida* toxin. *FEMS Microbiol. Lett.*, **43,** 45–51.

Frymus, T., Muller, E., Franz, B. and Petzoldt, K. (1989). Protection by toxoid-induced antibody of gnotobiotic piglets challenged with the dermonecrotic toxin of *Pasteurella multocida. Zentralblatt Fuer Veterinaermedizin Reihe B*, **36,** 674–680.

Fukuhara, S., Murga, C., Zohar, M., Igishi, T. and Gutkind, J.S. (1999). A novel PDZ domain containing guanine nucleotide exchange

factor links heterotrimeric G proteins to Rho. *J. Biol. Chem.*, **274,** 5868–5879.

Gillette, M.U., Buchanan, G.F., Artinian, L., Hamilton, S.E., Nathanson, N. M. and Liu, C. (2001). Role of the M1 receptor in regulating circadian rhythms. *Life Sci.*, **68,** 2467–2472.

Gosau, N., Fahimi-Vahid, M., Michalek, C., Schmidt, M. and Wieland, T. (2002). Signaling components involved in the coupling of alpha(1)-adrenoceptors to phospholipase D in neonatal rat cardiac myocytes. *Naunyn Schmiedebergs Arch. Pharmacol.*, **365,** 468–476.

Gratacap, M.P., Payrastre, B., Nieswandt, B. and Offermanns, S. (2001). Differential regulation of Rho and Rac through heterotrimeric G-proteins and cyclic nucleotides. *J. Biol. Chem.*, **276,** 47906–47913.

Gwaltney, S.M., Galvin, R.J., Register, K.B., Rimler, R.B. and Ackermann, M.R. (1997). Effects of *Pasteurella multocida* toxin on porcine bone marrow cell differentiation into osteoclasts and osteoblasts. *Vet. Pathol.*, **34,** 421–430.

Hamilton, T.D., Roe, J.M., Hayes, C.M. and Webster, A.J. (1998). Effect of ovalbumin aerosol exposure on colonization of the porcine upper airway by *Pasteurella multocida* and effect of colonization on subsequent immune function. *Clin. Diagn. Lab. Immunol.*, **5,** 494–498.

Harmey, D., Stenbeck, G., Nobes, C.D., Lax, A.J. and Grigoriadis, A.E. (2004). Regulation of osteoblast differentiation by *Pasteurella multocida* toxin (PMT): A role for Rho GTPase in bone formation. *J. Bone Mineral Res.*, **19,** 661–670.

Harris, D.L. and Switzer, W.P. (1968). Turbinate atrophy in young pigs exposed to *Bordetella bronchiseptica, Pasteurella multocida*, and combined inoculum. *Am. J. Vet. Res.*, **29,** 777–785.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A.G., Sternweis, P.C. and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. *Science*, **280,** 2112–2114.

Higgins, T.E., Murphy, A.C., Staddon, J.M., Lax, A.J. and Rozengurt, E. (1992). *Pasteurella multocida* toxin is a potent inducer of anchorage-independent cell growth. *Proc. Natl. Acad. Sci. USA*, **89,** 4240–4244.

Horiguchi, Y. (2001). *Escherichia coli* cytotoxic necrotizing factors and *Bordetella* dermonecrotic toxin: the dermonecrosis-inducing toxins activating Rho small GTPases. *Toxicon*, **39,** 1619–1627.

Horiguchi, Y., Inoue, N., Masuda, M., Kashimoto, T., Katahira, J., Sugimoto, N. and Matsuda, M. (1997). *Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln-63 of the GTP-binding protein Rho. *Proc. Natl. Acad. Sci. USA*, **94,** 11623–11626.

Horiguchi, Y., Senda, T., Sugimoto, N., Katahira, J. and Matsuda, M. (1995). *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein Rho. *J. Cell Sci.*, **108,** 3243–3251.

Horiguchi, Y., Sugimoto, N. and Matsuda, M. (1993). Stimulation of DNA synthesis in osteoblast-like MC3T3-E1 cells by *Bordetella bronchiseptica* dermonecrotic toxin. *Infect. Immun.*, **61,** 3611–3615.

Hoskins, I.C. and Lax, A.J. (1996). Constitutive expression of *Pasteurella multocida* toxin. *FEMS Microbiol. Lett.*, **141,** 189–193.

iDali, C., Foged, N.T., Frandsen, P.L., Nielsen, M.H. and Elling, F. (1991). Ultrastructural localization of the *Pasteurella multocida* toxin in a toxin-producing strain. *J. Gen. Microbiol.*, **137,** 1067–1071.

Il'ima, Z.M. and Zasukhin, M. (1975). Role of *Pasteurella* toxins in the pathogenesis of infectious atrophic rhinitis. *Sb. Nauchn. Rab. Sib. Nauchn. Issled. Inst. Omsk*, **25,** 76–86.

Jarvinen, L.Z., Hogenesch, H., Suckow, M.A. and Bowersock, T.L. (1998). Induction of protective immunity in rabbits by coadministration of inactivated *Pasteurella multocida* toxin and potassium thiocyanate extract. *Infect. Immun.*, **66,** 3788–3795.

Jordan, R.W., Hamilton, T.D., Hayes, C.M., Patel, D., Jones, P.H., Roe, J.M. and Williams, N.A. (2003). Modulation of the humoral immune response of swine and mice mediated by toxigenic *Pasteurella multocida*. *FEMS Immunol. Med. Microbiol.*, **39,** 51–59.

Jordan, R.W. and Roe, J.M. (2004). An experimental mouse model of progressive atrophic rhinitis of swine. *Vet. Microbiol.*, **103,** 201–207.

Jutras, I. and Martineau-Doize, B. (1996). Stimulation of osteoclast-like cell formation by *Pasteurella multocida* toxin from hemopoietic progenitor cells in mouse bone marrow cultures. *Can. J. Vet. Res.*, **60,** 34–39.

Kamp, E.M. and Kimman, T.G. (1988). Induction of nasal turbinate atrophy in germ-free pigs, using *Pasteurella multocida*, as well as bacterium-free crude and purified dermonecrotic toxin of P. multocida. *Am. J. Vet. Res.*, **49,** 1844–1849.

Kamp, E.M., Popma, J.K. and Van Leengoed, L.A. (1987). Serotyping of *Hemophilus pleuropneumoniae* in the Netherlands: with emphasis on heterogeneity within serotype 1 and (proposed) serotype 9. *Vet. Microbiol.*, **13,** 249–257.

Kamps, A.M., Kamp, E.M. and Smits, M.A. (1990). Cloning and expression of the dermonecrotic toxin gene of *Pasteurella multocida* ssp. *multocida* in *Escherichia coli*. *FEMS Microbiol. Lett.*, **55,** 187–190.

Katoh, H., Aoki, J., Yamaguchi, Y., Kitano, Y., Ichikawa, A. and Negishi, M. (1998). Constitutively active $G\alpha_{12}$, $G\alpha_{13}$, and $G\alpha_{q}$ induce Rho-dependent neurite retraction through different signaling pathways. *J. Biol. Chem.*, **273,** 28700–28707.

Kielstein, P. (1986). On the occurrence of toxin-producing *Pasteurella multocida* strains in atrophic rhinitis and in pneumonias of swine and cattle. *Zentralblatt Fuer Veterinaermedizin Reihe B*, **33,** 418–424.

Kielstein, P. and Elias, B. (1985). Zur Bedeutung von *Bordetella bronchiseptica* und *Pasteurella multocida* bei der Rhinitis atrophicans suum. *Zentralblatt Fuer Veterinaermedizin Reihe B*, **32,** 694–705.

Kimman, T.G., Lowik, C.W., van de Wee-Pals, L.J., Thesingh, C.W., Defize, P., Kamp, E.M. and Bijvoet, O.L. (1987). Stimulation of bone resorption by inflamed nasal mucosa, dermonecrotic toxin-containing conditioned medium from *Pasteurella multocida*, and purified dermonecrotic toxin from *P. multocida*. *Infect. Immun.*, **55,** 2110–2116.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G. and Sternweis, P.C. (1998). p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science*, **280,** 2109–2111.

Kurose, H. (2003). $G\alpha_{12}$ and $G\alpha_{13}$ as key regulatory mediator in signal transduction. *Life Sci.*, **74,** 155–161.

Lacerda, H.M., Lax, A.J. and Rozengurt, E. (1996). *Pasteurella multocida* toxin, a potent intracellularly acting mitogen, induces $p125^{FAK}$ and paxillin tyrosine phosphorylation, actin stress fiber formation, and focal contact assembly in Swiss 3T3 cells. *J. Biol. Chem.*, **271,** 439–445.

Lacerda, H.M., Pullinger, G.D., Lax, A.J. and Rozengurt, E. (1997). Cytotoxic necrotizing factor 1 from *Escherichia coli* and dermonecrotic toxin from *Bordetella bronchiseptica* induce p21(Rho)-dependent tyrosine phosphorylation of focal adhesion kinase and paxillin in Swiss 3T3 cells. *J. Biol. Chem.*, **272,** 9587–9596.

Lax, A.J. and Chanter, N. (1990). Cloning of the toxin gene from *Pasteurella multocida* and its role in atrophic rhinitis. *J. Gen. Microbiol.*, **136,** 81–87.

Lax, A.J., Chanter, N., Pullinger, G.D., Higgins, T., Staddon, J.M. and Rozengurt, E. (1990). Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*. *FEBS Lett.*, **277,** 59–64.

Lax, A.J. and Thomas, W. (2002). How bacteria could cause cancer: one step at a time. *Trends Microbiol.*, **10,** 293–299.

Lei, Q., Talley, E.M. and Bayliss, D.A. (2001). Receptor-mediated inhibition of G protein-coupled inwardly rectifying potassium channels involves $G\alpha_q$ family subunits, phospholipase C, and a readily diffusible messenger. *J. Biol. Chem.*, **276,** 16720–16730.

Lemichez, E., Flatau, G., Bruzzone, M., Boquet, P. and Gauthier, M. (1997). Molecular localization of the *Escherichia coli* cytotoxic necrotizing factor CNF1 cell-binding and catalytic domains. *Mol. Microbiol.*, **24,** 1061–1070.

Lockman, H.A., Gillespie, R.A., Baker, B.D. and Shakhnovich, E. (2002). *Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor. *Infect. Immun.*, **70,** 2708–2714.

Magyar, T., Chanter, N., Lax, A.J., Rutter, J.M. and Hall, G.A. (1988). The pathogenesis of turbinate atrophy in pigs caused by *Bordetella bronchiseptica. Vet. Microbiol.*, **18,** 135–146.

Magyar, T., King, V.L. and Kovacs, F. (2002). Evaluation of vaccines for atrophic rhinitis—a comparison of three challenge models. *Vaccine*, **20,** 1797–1802.

Martineau-Doize, B., Caya, I., Gagne, S., Jutras, I. and Dumas, G. (1993). Effects of *Pasteurella multocida* toxin on the osteoclast population of the rat. *J. Comp. Pathol.*, **108,** 81–91.

Masuda, M., Minami, M., Shime, H., Matsuzawa, T. and Horiguchi, Y. (2002). *In vivo* modifications of small GTPase Rac and Cdc42 by *Bordetella* dermonecrotic toxin. *Infect. Immun.*, **70,** 998–1001.

Matsuzawa, T., Kashimoto, T., Katahira, J. and Horiguchi, Y. (2002). Identification of a receptor-binding domain of *Bordetella* dermonecrotic toxin. *Infect. Immun.*, **70,** 3427–3432.

May, B.J., Zhang, Q., Li, L.L., Paustian, M.L., Whittam, T.S. and Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proc. Natl. Acad. Sci. USA*, **98,** 3460–3465.

Meyer, T., Wellner-Kienitz, M.C., Biewald, A., Bender, K., Eickel, A. and Pott, L. (2001). Depletion of phosphatidylinositol 4,5-bisphosphate by activation of phospholipase C-coupled receptors causes slow inhibition but not desensitization of G protein-gated inward rectifier $K^+$ current in atrial myocytes. *J. Biol. Chem.*, **276,** 5650–5658.

Meysick, K.C., Mills, M. and O'Brien, A.D. (2001). Epitope mapping of monoclonal antibodies capable of neutralizing cytotoxic necrotizing factor type 1 of uropathogenic *Escherichia coli. Infect. Immun.*, **69,** 2066–2074.

Mullan, P.B. and Lax, A.J. (1996). *Pasteurella multocida* toxin is a mitogen for bone cells in primary culture. *Infect. Immun.*, **64,** 959–965.

Mullan, P.B. and Lax, A.J. (1998). *Pasteurella multocida* toxin stimulates bone resorption by osteoclasts via interaction with osteoblasts. *Calcif. Tissue Int.*, **63,** 340–345.

Murphy, A.C. and Rozengurt, E. (1992). *Pasteurella multocida* toxin selectively facilitates phosphatidylinositol 4,5-bisphosphate hydrolysis by bombesin, vasopressin, and endothelin. Requirement for a functional G protein. *J. Biol. Chem.*, **267,** 25296–25303.

Nakai, T. and Kume, K. (1987). Purification of three fragments of the dermonecrotic toxin from *Pasteurella multocida. Res. Vet. Sci.*, **42,** 232–237.

Nakai, T., Kume, K., Yoshikawa, H., Oyamada, T. and Yoshikawa, T. (1986). Changes in the nasal mucosa of specific pathogen-free neonatal pigs infected with *Pasteurella multocida* or *Bordetella bronchiseptica. Nippon Juigaku Zasshi -Jap. J. Vet. Sci.*, **48,** 693–701.

Nakai, T., Sawata, A. and Kume, K. (1985). Intracellular locations of dermonecrotic toxins in *Pasteurella multocida* and in *Bordetella bronchiseptica. Am. J. Vet. Res.*, **46,** 870–874.

Nakai, T., Sawata, A., Tsuji, M. and Kume, K. (1984a). Characterization of dermonecrotic toxin produced by serotype D strains of *Pasteurella multocida. Am. J. Vet. Res.*, **45,** 2410–2413.

Nakai, T., Sawata, A., Tsuji, M., Samejima, Y. and Kume, K. (1984b). Purification of dermonecrotic toxin from a sonic extract of *Pasteurella multocida* SP-72 serotype D. *Infect. Immun.*, **46,** 429–434.

Nielsen, J.P., Foged, N.T., Sorensen, V., Barfod, K., Bording, A. and Petersen, S. K. (1991). Vaccination against progressive atrophic rhinitis with a recombinant *Pasteurella multocida* toxin derivative. *Can. J. Vet. Res.*, **55,** 128–138.

Nielsen, J.P. and Rosdahl, V.T. (1990). Development and epidemiological applications of a bacteriophage typing system for typing *Pasteurella multocida. J. Clin. Microbiol.*, **28,** 103–107.

Ohnishi, T., Horiguchi, Y., Masuda, M., Sugimoto, N. and Matsuda, M. (1998). *Pasteurella multocida* toxin and *Bordetella bronchiseptica* dermonecrotizing toxin elicit similar effects on cultured cells by different mechanisms. *J. Vet. Med. Sci.*, **60,** 301–305.

Okada, Y., Fujiyama, R., Miyamoto, T. and Sato, T. (2001). Saccharin activates cation conductance via inositol 1,4,5-trisphosphate production in a subset of isolated rod taste cells in the frog. *Eur. J. Neurosci.*, **13,** 308–314.

Orth, J.H., Blocker, D. and Aktories, K. (2003). His1205 and His1223 are essential for the activity of the mitogenic *Pasteurella multocida* toxin. *Biochem.*, **42,** 4971–4977.

Orth, J.H., Lang, S. and Aktories, K. (2004). Action of *Pasteurella multocida* toxin depends on the helical domain of $G\alpha_q$. *J. Biol. Chem.*, **279,** 34150–34155.

Oswald, E., Sugai, M., Labigne, A., Wu, H.C., Fiorentini, C., Boquet, P. and O'Brien, A.D. (1994). Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. *Proc. Natl. Acad. Sci. USA*, **91,** 3814–3818.

Pedersen, K.B. and Barfod, K. (1981). The etiological significance of *Bordetella bronchiseptica* and *Pasteurella multocida* in atrophic rhinitis of swine. *Nordisk Veterinaermedicin*, **33,** 513–522.

Pedersen, K.B. and Barfod, K. (1982). Effect on the incidence of atrophic rhinitis of vaccination of sows with a vaccine containing *Pasteurella multocida* toxin. *Nordisk Veterinaermedicin*, **34,** 293–302.

Pedersen, K.B. and Elling, F. (1984). The pathogenesis of atrophic rhinitis in pigs induced by toxigenic *Pasteurella multocida*. *J. Comp. Pathol.*, **94,** 203–214.

Pedersen, K.B., Nielsen, J.P., Foged, N.T., Elling, F., Nielsen, N.C. and Willeberg, P. (1988). Atrophic rhinitis in pigs: proposal for a revised definition. *Vet. Record*, **122,** 190–191.

Pei, S., Doye, A. and Boquet, P. (2001). Mutation of specific acidic residues of the CNF1 T domain into lysine alters cell membrane translocation of the toxin. *Mol. Microbiol.*, **41,** 1237–1247.

Pennings, A.M. and Storm, P.K. (1984). A test in vero cell monolayers for toxin production by strains of *Pasteurella multocida* isolated from pigs suspected of having atrophic rhinitis. *Vet. Microbiol.*, **9,** 503–508.

Petersen, S.K. (1990). The complete nucleotide sequence of the *Pasteurella multocida* toxin gene and evidence for a transcriptional repressor, TxaR. *Mol. Microbiol.*, **4,** 821–830.

Petersen, S.K. and Foged, N.T. (1989). Cloning and expression of the *Pasteurella multocida* toxin gene, *toxA*, in *Escherichia coli*. *Infect. Immun.*, **57,** 3907–3913.

Petersen, S.K., Foged, N.T., Bording, A., Nielsen, J.P., Riemann, H.K. and Frandsen, P.L. (1991). Recombinant derivatives of *Pasteurella multocida* toxin: candidates for a vaccine against progressive atrophic rhinitis. *Infect. Immun.*, **59,** 1387–1393.

Pettit, R.K., Ackermann, M.R. and Rimler, R.B. (1993a). Receptor-mediated binding of *Pasteurella multocida* dermonecrotic toxin to canine osteosarcoma and monkey kidney (vero) cells. *Lab. Invest.*, **69,** 94–100.

Pettit, R.K., Rimler, R.B. and Ackermann, M.R. (1993b). Protection of *Pasteurella multocida* dermonecrotic toxin-challenged rats by toxoid-induced antibody. *Vet. Microbiol.*, **34,** 167–173.

Pullinger, G.D., Adams, T.E., Mullan, P.B., Garrod, T.I. and Lax, A.J. (1996). Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of *Bordetella* spp. *Infect. Immun.*, **64,** 4163–4171.

Pullinger, G.D., Bevir, T. and Lax, A.J. (2004). The *Pasteurella multocida* toxin is encoded within a lysogenic bacteriophage. *Mol. Microbiol*., **51,** 255–269.

Pullinger, G.D., Sowdhamini, R. and Lax, A.J. (2001). Localization of functional domains of the mitogenic toxin of *Pasteurella multocida*. *Infect. Immun*., **69,** 7839–7850.

Rajeev, S., Nair, R. V., Kania, S. A. and Bemis, D.A. (2003). Expression of a truncated *Pasteurella multocida* toxin antigen in *Bordetella bronchiseptica*. *Vet. Microbiol*., **94,** 313–323.

Riising, H.J., van Empel, P. and Witvliet, M. (2002). Protection of piglets against atrophic rhinitis by vaccinating the sow with a vaccine against *Pasteurella multocida* and *Bordetella bronchiseptica*. *Vet. Record*, **150,** 569–571.

Roop, R.M., 2nd, Veit, H. P., Sinsky, R.J., Veit, S.P., Hewlett, E.L. and Kornegay, E. T. (1987). Virulence factors of *Bordetella bronchiseptica* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. *Infect. Immun*., **55,** 217–222.

Rosendal, S., Frandsen, P.L., Nielsen, J.P. and Gallily, R. (1995). *Pasteurella multocida* toxin induces IL-6, but not IL-1α or TNFα in fibroblasts. *Can. J. Vet. Res*., **59,** 154–156.

Rozengurt, E., Higgins, T., Chanter, N., Lax, A.J. and Staddon, J.M. (1990). *Pasteurella multocida* toxin: potent mitogen for cultured fibroblasts. *Proc. Natl. Acad. Sci. USA*, **87,** 123–127.

Rutter, J.M. (1981). Quantitative observations on *Bordetella bronchiseptica* infection in atrophic rhinitis of pigs. *Vet. Record*, **108,** 451–454.

Rutter, J.M. (1983). Virulence of *Pasteurella multocida* in atrophic rhinitis of gnotobiotic pigs infected with *Bordetella bronchiseptica*. *Res. Vet. Sci*., **34,** 287–295.

Rutter, J.M. (1985). Atrophic rhinitis in swine. *Adv. Vet. Sci. Comp. Med*., **29,** 239–279.

Rutter, J.M., Francis, L.M. and Sansom, B.F. (1982). Virulence of *Bordetella bronchiseptica* from pigs with or without atrophic rhinitis. *J. Med. Microbiol*., **15,** 105–116.

Rutter, J.M. and Luther, P.D. (1984). Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis of pigs. *Vet. Record*, **114,** 393–396.

Rutter, J.M. and Mackenzie, A. (1984). Pathogenesis of atrophic rhinitis in pigs: a new perspective. *Vet. Record*, **114,** 89–90.

Rutter, J.M., Taylor, R.J., Crighton, W.G., Robertson, I.B. and Benson, J. A. (1984). Epidemiological study of *Pasteurella multocida* and *Bordetella bronchiseptica* in atrophic rhinitis. *Vet. Record*, **115,** 615–619.

Sabri, A., Pak, E., Alcott, S.A., Wilson, B.A. and Steinberg, S. F. (2000). Coupling function of endogenous alpha(1)- and beta-adrenergic receptors in mouse cardiomyocytes. *Circ. Res*., **86,** 1047–1053.

Sabri, A., Wilson, B.A. and Steinberg, S. F. (2002). Dual actions of the $G\alpha_q$ agonist Pasteurella multocida toxin to promote cardiomyocyte hypertrophy and enhance apoptosis susceptibility. *Circ. Res*., **90,** 850–857.

Sah, V.P., Seasholtz, T.M., Sagi, S. A. and Brown, J.H. (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annu. Rev. Pharmacol. Toxicol*., **40,** 459–489.

Sakano, T., Okada, M., Taneda, A., Mukai, T. and Sato, S. (1997). Effect of *Bordetella bronchiseptica* and serotype D *Pasteurella multocida* bacterin-toxoid on the occurrence of atrophic rhinitis after experimental infection with *B. bronchiseptica* and toxigenic type A *P. multocida*. *J. Vet. Med. Sci*., **59,** 55–57.

Sakano, T., Taneda, A., Okada, M., Ono, M., Hayashi, Y. and Sato, S. (1992). Toxigenic type A *Pasteurella multocida* as a causative agent of nasal turbinate atrophy in swine. *J. Vet. Med. Sci*., **54,** 403–407.

Schmidt, G., Goehring, U.M., Schirmer, J., Uttenweiler-Joseph, S., Wilm, M., Lohmann, M., Giese, A., Schmalzing, G. and Aktories, K. (2001). Lysine and polyamines are substrates for transglutamination of Rho by the *Bordetella* dermonecrotic toxin. *Infect. Immun*., **69,** 7663–7670.

Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M. and Aktories, K. (1997). Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. *Nature*, **387,** 725–729.

Schmidt, G., Selzer, J., Lerm, M. and Aktories, K. (1998). The Rho-deamidating cytotoxic necrotizing factor 1 from *Escherichia coli* possesses transglutaminase activity. Cysteine 866 and histidine 881 are essential for enzyme activity. *J. Biol. Chem*., **273,** 13669–13674.

Seo, B., Choy, E.W., Maudsley, S., Miller, W.E., Wilson, B.A. and Luttrell, L.M. (2000). *Pasteurella multocida* toxin stimulates mitogen-activated protein kinase via $G_{q/11}$-dependent transactivation of the epidermal growth factor receptor. *J. Biol. Chem*., **275,** 2239–2245.

Shime, H., Ohnishi, T., Nagao, K., Oka, K., Takao, T. and Horiguchi, Y. (2002). Association of *Pasteurella multocida* toxin with vimentin. *Infect. Immun*., **70,** 6460–6463.

Sleight, S., Wilson, B.A., Heimark, D.B. and Larner, J. (2002). $G_{q/11}$ is involved in insulin-stimulated inositol phosphoglycan putative mediator generation in rat liver membranes: co-localization of $G_{q/11}$ with the insulin receptor in membrane vesicles. *Biochem. Biophys. Res. Comm*., **295,** 561–569.

Smyth, M.G., Pickersgill, R.W. and Lax, A.J. (1995). The potent mitogen *Pasteurella multocida* toxin is highly resistant to proteolysis but becomes susceptible at lysosomal pH. *FEBS Lett*., **360,** 62–66.

Smyth, M.G., Sumner, I.G. and Lax, A.J. (1999). Reduced pH causes structural changes in the potent mitogenic toxin of *Pasteurella multocida*. *FEMS Microbiol. Lett*., **180,** 15–20.

Staddon, J.M., Barker, C.J., Murphy, A.C., Chanter, N., Lax, A.J., Michell, R. H. and Rozengurt, E. (1991a). *Pasteurella multocida* toxin, a potent mitogen, increases inositol 1,4,5-trisphosphate and mobilizes $Ca^{2+}$ in Swiss 3T3 cells. *J. Biol. Chem*., **266,** 4840–4847.

Staddon, J.M., Bouzyk, M.M. and Rozengurt, E. (1991b). A novel approach to detect toxin-catalyzed ADP-ribosylation in intact cells: its use to study the action of *Pasteurella multocida* toxin. *J. Cell Biol*., **115,** 949–958.

Staddon, J.M., Chanter, N., Lax, A.J., Higgins, T.E. and Rozengurt, E. (1990). *Pasteurella multocida* toxin, a potent mitogen, stimulates protein kinase C-dependent and -independent protein phosphorylation in Swiss 3T3 cells. *J. Biol. Chem*., **265,** 11841–11848.

Sterner-Kock, A., Lanske, B., Uberschar, S. and Atkinson, M.J. (1995). Effects of the *Pasteurella multocida* toxin on osteoblastic cells *in vitro*. *Vet. Pathol*., **32,** 274–279.

Suckow, M.A. (2000). Immunization of rabbits against *Pasteurella multocida* using a commercial swine vaccine. *Lab. Animals*, **34,** 403–408.

Suckow, M.A., Bowersock, T.L., Nielsen, K., Chrisp, C.E., Frandsen, P. L. and Janovitz, E. B. (1995). Protective immunity to *Pasteurella multocida* heat-labile toxin by intranasal immunization in rabbits. *Lab. Animal Sci*., **45,** 526–532.

Suckow, M.A., Chrisp, C.E. and Foged, N.T. (1991). Heat-labile, toxin-producing isolates of *Pasteurella multocida* from rabbits. *Lab. Animal Sci*., **41,** 151–156.

Switzer, W.P. (1956). Studies on infectious atrophic rhinitis. V. Concept that several agents may cause turbinate atrophy. *Am. J. Vet. Res*., **17,** 478–484.

Thomas, W., Pullinger, G.D., Lax, A.J. and Rozengurt, E. (2001). *Escherichia coli* cytotoxic necrotizing factor and *Pasteurella multocida* toxin induce focal adhesion kinase autophosphorylation and Src association. *Infect. Immun*., **69,** 5931–5935.

Thurston, J.R., Rimler, R.B., Ackermann, M.R. and Cheville, N.F. (1992). Use of rats to compare atrophic rhinitis vaccines for protection against effects of heat-labile protein toxin produced by *Pasteurella multocida* serogroup D. *Vet. Immunol. Immunopathol*., **33,** 155–162.

Thurston, J.R., Rimler, R.B., Ackermann, M.R., Cheville, N.F. and Sacks, J.M. (1991). Immunity induced in rats vaccinated with toxoid prepared from heat-labile toxin produced by *Pasteurella multocida* serogroup D. *Vet. Microbiol*., **27,** 169–174.

Tornoe, N. and Nielsen, N.C. (1976). Inoculation experiments with *Bordetella bronchiseptica* strains in SPF pigs. *Nordisk Veterinaermedicin*, **28,** 233–242.

Umemori, H., Hayashi, T., Inoue, T., Nakanishi, S., Mikoshiba, K. and Yamamoto, T. (1999). Involvement of protein tyrosine phosphatases in activation of the trimeric G protein $G_{q/11}$. *Oncogene*, **18,** 7399–7402.

Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K. and Yamamoto, T. (1997). Activation of the G protein $G_{q/11}$ through tyrosine phosphorylation of the α subunit. *Science*, **276,** 1878–1881.

van Diemen, P.M., de Jong, M.F., de Vries Reilingh, G., van der Hel, P. and Schrama, J.W. (1994a). Intranasal administration of *Pasteurella multocida* toxin in a challenge-exposure model used to induce subclinical signs of atrophic rhinitis in pigs. *Am. J. Vet. Res.*, **55,** 49–54.

van Diemen, P.M., de Vries Reilingh, G. and Parmentier, H.K. (1994b). Immune responses of piglets to *Pasteurella multocida* toxin and toxoid. *Vet. Immunol. Immunopathol.*, **41,** 307–321.

van Diemen, P. M., de Vries Reilingh, G. and Parmentier, H. K. (1996). Effect of *Pasteurella multocida* toxin on *in vivo* immune responses in piglets. *Vet. Quarterly*, **18,** 141–146.

Vogt, S., Grosse, R., Schultz, G. and Offermanns, S. (2003). Receptor-dependent RhoA activation in $G_{12}/G_{13}$-deficient cells: genetic evidence for an involvement of $G_q/G_{11}$. *J. Biol. Chem.*, **278,** 28743–28749.

Walker, K.E. and Weiss, A.A. (1994). Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. *Infect. Immun.*, **62,** 3817–3828.

Ward, P.N., Higgins, T. E., Murphy, A.C., Mullan, P.B., Rozengurt, E. and Lax, A.J. (1994). Mutation of a putative ADP-ribosylation motif in the *Pasteurella multocida* toxin does not affect mitogenic activity. *FEBS Lett.*, **342,** 81–84.

Ward, P.N., Miles, A.J., Sumner, I.G., Thomas, L.H. and Lax, A.J. (1998). Activity of the mitogenic *Pasteurella multocida* toxin requires an essential C-terminal residue. *Infect. Immun.*, **66,** 5636–5642.

Wilson, B.A., Aminova, L.R., Ponferrada, V.G. and Ho, M. (2000). Differential modulation and subsequent blockade of mitogenic signaling and cell cycle progression by *Pasteurella multocida* toxin. *Infect. Immun.*, **68,** 4531–4538.

Wilson, B.A., Ponferrada, V.G., Vallance, J.E. and Ho, M. (1999). Localization of the intracellular activity domain of *Pasteurella multocida* toxin to the N terminus. *Infect. Immun.*, **67,** 80–87.

Wilson, B.A., Zhu, X., Ho, M. and Lu,L. (1997). *Pasteurella multocida* toxin activates the inositol triphosphate signaling pathway in *Xenopus* oocytes via $G_q$α-coupled phospholipase C-β1. *J. Biol. Chem.*, **272,** 1268–1275.

Wu, D., Katz, A. and Simon, M.I. (1993). Activation of phospholipase C β2 by the α and βγ subunits of trimeric GTP-binding protein. *Proc. Natl. Acad. Sci. USA*, **90,** 5297–5301.

Zimmerman, T.E., Deeb, B.J. and DiGiacomo, R.F. (1992). Polypeptides associated with *Pasteurella multocida* infection in rabbits. *Am. J. Vet. Res.*, **53,** 1108–1112.

Zywietz, A., Gohla, A., Schmelz, M., Schultz, G. and Offermanns, S. (2001). Pleiotropic effects of *Pasteurella multocida* toxin are mediated by $G_q$-dependent and -independent mechanisms. Involvement of $G_q$ but not $G_{11}$. *J. Biol. Chem.*, **276,** 3840–3845.

CHAPTER

# 23

# Cytolethal distending toxins

*Monica Thelestam and Teresa Frisan*

## INTRODUCTION

The first cytolethal distending toxin (CDT) was reported in 1987 as a novel type of toxin activity produced by pathogenic strains of *Escherichia coli*. The major hallmark of the observed cytotoxic effect was a remarkable cell distention, evident 3–5 days after addition of bacterial culture supernatants to cells growing *in vitro*, and resulting after a few more days in cell death (Johnson and Lior, 1987a). The same authors later identified a similar activity in *Shigella* (Johnson and Lior, 1987b) and *Campylobacter* spp (Johnson and Lior, 1988a), and they designated this putative toxin as "cytolethal distending toxin" (CDT), based on the conspicuous morphological effect (Figure 23.1).

It is now clear that the CDTs are produced from three linked genes, which are designated *cdtA*, *cdtB*, and *cdtC* and encode the proteins (CdtA, CdtB, CdtC) (Figure 23.2) with predicted molecular masses of around 25, 30, and 20 kDa, respectively. Several Gram-negative bacteria have been shown to produce CDTs, such as *Shigella spp* (Okuda *et al*., 1995), *Campylobacter jejuni* (Pickett *et al*., 1996), *Haemophilus ducreyi* (Cope *et al*., 1997), *Actinobacillus actinomycetemcomitans* (Mayer *et al*., 1999; Sugai *et al*., 1998), and several *Helicobacter spp* (Chien *et al*., 2000; Kostia *et al*., 2003; Taylor *et al*., 2003; Young *et al*., 2000b). So far no Gram-positive bacterium is known to produce CDT. The CDT-producing bacteria are listed in Table 23.1.

The CDT gene products have no immediate resemblance to any other known proteins, but using the PSI-BLAST search technique, a DNase I–like enzymatic activity could be identified in the CdtB component of CDTs from *E. coli* and *C. jejuni*, respectively (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000). The proposed DNA damaging effect was consistent with parallel observations on the *Haemophilus ducreyi* CDT, as the cellular responses to this CDT were identical to the cell cycle checkpoint responses evoked by ionizing radiation (IR), a well-known DNA damaging agent (Cortes-Bratti *et al*., 2001b). Indeed, we could show later that the *Haemophilus ducreyi* CDT (HdCDT) induces DNA double-strand breaks (DSBs) upon natural intoxication of mammalian cells (Frisan *et al*., 2003), thus attaining direct proof for the CDT attack on DNA. Moreover, the recently solved crystal structure of the HdCDT holotoxin revealed that this toxin consists of an enzyme belonging to the DNase I family (CdtB), which is associated with two ricin-like lectin domains (CdtA and CdtC) (Nesic *et al*., 2004).

Even though all CDTs have the same enzymatic activity, the CDT variants produced by different bacterial species might vary with regard to receptor specificity and internalization pathway. Therefore, we have proposed a nomenclature to avoid a confusing flora of different names in the literature on CDTs (Cortes-Bratti *et al*., 2001a). A particular CDT is specified exactly by indicating the initials of the producing bacterium before CDT and, if necessary, the strain number or other common designation after CDT (e.g., HdCDT: *H. ducreyi* CDT or EcCDT-III: *E. coli* CDT type III), as indicated in Table 23.1.

All previously known bacterial protein toxins attack either the plasma membrane or various targets located in the cytosol. The CDTs represent a completely novel concept among bacterial toxins, as they act instead on DNA. Therefore, this exciting family of toxins raises interesting questions that are novel to the field of toxinology. For instance, how does the active subunit of CDT reach its target, i.e., which is the molecular

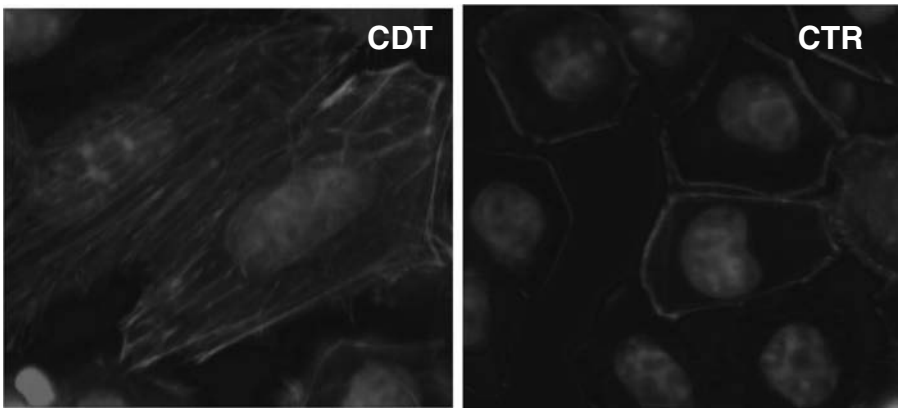



**FIGURE 23.1** CDT intoxication induces cell distention and promotion of actin stress fibers. HeLa cells were either left untreated (CTR) or exposed to HdCDT (CDT, 2μg/ml), and further incubated for 24h. F-actin was stained with FITC-phalloidin, and nuclei were counterstained with Hoechst 33258.

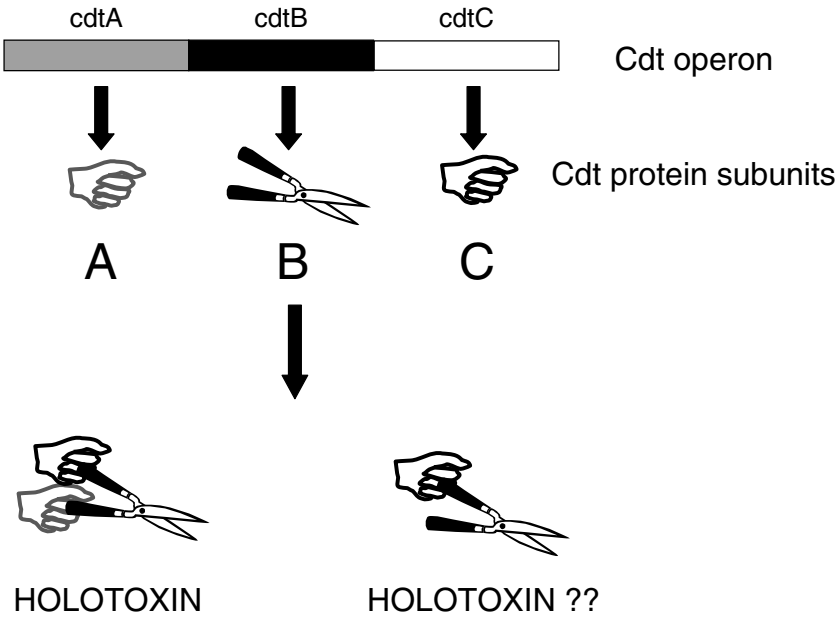



**FIGURE 23.2** Schematic representation of the three linked CDT genes and the respective protein subunits. CdtB is the active subunit, which causes DNA damage. CdtA and CdtC are required for binding the holotoxin to the plasma membrane of the target cell. Although the tripartite holotoxin (CdtA+CdtB+CdTC) has been found to be the most potent inducer of cellular intoxication, some doubts still exist as to whether this is the only possible subunit combination. This issue is discussed in the text.

mechanism for the transport of CdtB to the nucleus? Which are the molecular details for the action of CDT on DNA and which are the cellular responses, both immediate and long-term, to this unusual action of a bacterial protein toxin? Because of the genotoxic activity, the possibility exists that CDT could be disease related, not only as a cytotoxic agent in the pathogenesis of infections, but also in the long term as a potentially carcinogenic agent. However, the same genotoxic activity also makes this family of toxins particularly interesting as a basis for immunotoxins with good potential for anti-tumor activity.

This chapter will deal with several aspects of CDTs, but since this toxin family is relatively young, the main focus will be on their molecular action on mammalian cells. We will follow the toxin from the moment it meets the plasma membrane of a target cell until it reaches its target, and we will discuss how various types of cells respond to the toxin attack. The possible pathogenic roles of CDTs will also be discussed briefly, as well as potential practical applications of CDTs in the biomedical field.

## ACTION OF CDT ON MAMMALIAN CELLS

### The players: functions of the three different CDT subunits

Early genetic studies demonstrated that all three *cdt* genes need to be expressed in the producing bacterium for the generation of active (cytotoxic) CDT (Cope *et al.*, 1997; Pickett *et al.*, 1994; Pickett *et al.*, 1996; Sugai *et al.*, 1998; Young *et al.*, 2000b). However, the difficulty in

**TABLE 23.1** Distribution of CDT genes in bacterial species and nomenclature

| Species | Gene(s) detected | CDT activity | Designation | References |
|---|---|---|---|---|
| *Escherichia coli* | *cdtABC* | + | EcCDTI | (Scott and Kaper, 1994) |
| *E. coli* | *cdtABC* | + | EcCDTII | (Pickett *et al.*, 1994) |
| *E. coli* | *cdtABC* | + | EcCDTIII | (Peres *et al.*, 1997) |
| *E. coli* | *cdtABC* | + | EcCDTIV | (Toth *et al.*, 2003) |
| *Shigella dysenteriae* | *cdtABC* | + | SdCDT | (Okuda *et al.*, 1995) |
| *S. boydii* | – | + | SbCDT | (Okuda *et al.*, 1995) |
| *S. sonnei* | *cdtA* | nr | SsCdtA | (Okuda *et al.*, 1995) |
| *Haemophilus ducreyi* | *cdtABC* | + | HdCDT | (Cope *et al.*, 1997) |
| *Actinobacillus actinomycetemcomitans* | *cdtABC* | + | AaCDT | (Mayer *et al.*, 1999; Sugai *et al.*, 1998) |
| *Helicobacter hepaticus* | *cdtABC* | + | HhCdtB | (Young *et al.*, 2000a) |
| *H. pullorum* | *cdtB* | + | HpuCdtB | (Young *et al.*, 2000b) |
| *H. bilis* | *cdtB* | + | HbCdtB | (Chien *et al.*, 2000) |
| *H. canis* | *cdtB* | + | HcaCdtB | (Chien *et al.*, 2000) |
| *H. flexispira* | *cdtB* | + | HfCdtB | (Kostia *et al.*, 2003) |
| *H. cinaedi* | *cdtB* | + | HciCdtB | (Taylor *et al.*, 2003) |
| *Helicobacter sp (strain 98-6070)* | *cdtB* | + | HspCdtB I | (Chien *et al.*, 2000) |
| *Helicobacter spp (strain 96-1001)* | *cdtB* | – | HspCdtB II | (Chien *et al.*, 2000) |
| *Campylobacter jejuni* | *cdtABC* | + | CjCDT | (Pickett *et al.*, 1996) |
| *C. coli* | *cdtABC* | + | CcCdtB | (Pickett *et al.*, 1996) |
| *C. fetus* | *cdtB*[a] | + | CfCdtB | (Pickett *et al.*, 1996) |
| *C. hyointestinalis* | *cdtB*[a] | + | ChCdtB | (Pickett *et al.*, 1996) |
| *C. lari* | *cdtB*[a] | + | ClCdtB | (Pickett *et al.*, 1996) |
| *C."upsaliensis"* | *cdtB*[a] | + | CuCdtB | (Pickett *et al.*, 1996) |
| *Salmonella typhi* | *cdtB* | +[b] | StCdtB | (Haghjoo and Galan, 2004) |

nr: not reported
[a]: probable presence of *cdtB* sequences
[b]: activity observed only when *S. typhi* is internalized by the host cell

obtaining highly purified separate subunits from bacteria, which possess the entire *cdt* operon, created some confusion regarding the roles of the different subunits. Current studies are usually performed with recombinant CDT, but some discrepancies still remain and clarifying the roles of the subunits in cellular intoxication by CDT constitutes one of the most important frontiers in current CDT research. Therefore, we will start with a brief discussion of each subunit to set the scene for the ensuing journey from plasma membrane to nuclear target.

### *CdtB*

As stated previously, CdtB is definitely the most important active subunit showing DNase activity *in vitro* and *in vivo*. CdtB of various origins is cytotoxic after microinjection (Lara-Tejero and Galan, 2000) or electroporation/facilitated entry into cells (Elwell *et al.*, 2001; Mao and DiRienzo, 2002; Yamamoto *et al.*, 2004). In contrast, extracellularly added CdtB is not able to bind to most target cells and consequently is not cytotoxic by itself. Although the CdtB subunit of the AaCDT alone could cause G2 arrest in PHA-activated human T cells (Shenker *et al.*, 2000; Shenker *et al.*, 1999), the same authors later demonstrated that the presence of both the CdtA and CdtC subunits was required to achieve maximum cell cycle arrest in Jurkat cells (Shenker *et al.*, 2004). A holotoxin comprised of CdtABC was more than 50,000-fold more toxic to these cells than preparations containing either CdtAB or CdtBC. Thus, there is general consensus today that all three subunits must be added together for extracellularly induced maximal intoxication (Lara-Tejero and Galan, 2001; Lee *et al.*, 2003).

### *CdtA*

Shenker and coworkers (Shenker *et al.*, 2004) reported that a monoclonal antibody against CdtC co-precipitated CdtA and CdtB from extracts of CDT-producing *A. actinomycetemcomitans*. Interestingly, only a truncated form of CdtA (18 kDa) was detectable within this complex, suggesting that CdtA may undergo processing during the holotoxin assembly. The expression of two immunoreactive CdtA proteins corresponding to 25 and 18 kDa has been observed also for the HdCDT (Frisk *et al.*, 2001). CdtA seems to be able to bind to target cells, but alone it lacks cytotoxic activity (Lee *et al.*, 2003; Mao and DiRienzo, 2002). Also, the combination

of only CdtA and CdtB was without cytotoxicity on HeLa cells (Lara-Tejero and Galan, 2001; Lee *et al*., 2003). It is unclear whether the CdtA in these cases was in the truncated form or not.

### *CdtC*

CdtC has also been reported to be somehow processed in the holotoxin environment. The isoelectric point (pI) of the HdCdtC component was 1.5 pH units higher in recombinant strains expressing all three components than in recombinant strains expressing the CdtC protein alone (Frisk *et al*., 2001). A similar change of pI occurred after mixing the three individual recombinant components *in vitro*. Thus, it was suggested that HdCdtA/B may exert some kind of processing activity on HdCdtC, rendering it active (Deng *et al*., 2001; Frisk *et al*., 2001). Recently, however, the purified recombinant His-tagged AaCdtC alone, delivered to the cytosol with a lipid protein carrier, was found able to induce cell distention and eventually the death of CHO cells (Mao and DiRienzo, 2002). The specific mechanism of this putative CdtC cytotoxicity has not been elucidated.

### *Cytotoxicity of combined CdtB and CdtC*

Mao and DiRienzo (Mao and DiRienzo, 2002) also found an additive cytotoxic effect exerted by the combination of AaCdtB and AaCdtC when delivered into the cytosol together. We previously observed cytotoxicity with an extracellularly added, highly purified preparation of HdCDT in which we were not able to detect CdtA by Western-blot analysis (Li *et al*., 2002). This is consistent with the observation that extracts from *H. ducreyi* producing CDT in which the *cdtA* gene was mutated still had some cytotoxic activity (Lewis *et al*., 2001). Also, other workers reported the combination of purified HdCdtB with HdCdtC to be slightly cytotoxic (Wising *et al*., 2002). Likewise, a mixture of recombinant AaCdtB and AaCdtC could induce cell cycle arrest in HEp-2 cells, as measured by FACS analysis 72 h after toxin exposure (Akifusa *et al*., 2001). Similarly, the combined recombinant subunits CjCdtB and CjCdtC were able to induce G2 arrest in HeLa cells upon extracellular addition (Lee *et al*., 2003). On a microgram basis, however, this combination was only about 25% as effective as the tripartite CjCDT holotoxin. It would be of interest to determine the toxin amount produced by the bacteria *in vivo*, in order to understand whether the CdtB/C combination could have any physiological relevance.

In conclusion, the most reasonable interpretation of the combined current data on the CDT subunits is that CDT functions as an AB-toxin, where the DNase CdtB constitutes the active subunit, and a CdtAC heterodimer constitutes the optimal cell membrane-binding subunit. This tripartite holotoxin is the subunit combination with maximal activity in all tested cell systems. Apparently, the CdtC subunit alone can also function as a binding subunit, facilitating the cellular entry of CdtB, albeit with a much lower efficiency than the heterodimeric binding unit. That the AaCdtB alone can act on Jurkat cells (Shenker *et al*., 2004; Shenker *et al*., 2000) may have to do with specific properties of T cells, which in contrast to epithelial cells or fibroblasts appear able to bind and take up CdtB. The CDT binding process will be discussed in more detail in the next section.

## Pathway for the cellular internalization of CDT

Since CDT acts on DNA, it is conceivable that this toxin, like other intracellularly acting toxins, after binding to the cell surface has to be internalized and transported inside the target cell to the nucleus before it can act. In this section, we will discuss some recent studies relevant for both the binding and the internalization processes.

### *Cell surface binding of CDT subunits*

Different CDTs have a documented ability to intoxicate cells even after exposure times as short as 2 to 15 minutes (Aragon *et al*., 1997; Cortes-Bratti *et al*., 1999), and HdCDT could be absorbed out from the medium by repeatedly passing the same toxin solution over fresh HEp-2 cell cultures (Frisk *et al*., 2001). Such functional studies indirectly suggested that CDTs bind to cells rapidly and irreversibly, although no specificity of the binding could be demonstrated. It was earlier pointed out that a region in the CjCdtA, encompassing the amino acids 160 through 220, exhibits a lectin fold, which is also present in the binding subunits of the plant toxins ricin and abrin (Lara-Tejero and Galan, 2001). The recently determined crystal structure of HdCDT reinforced this observation and underscored the presence of a ricin-like lectin domain also in HdCdtC.

When recombinant HdCdtA and HdCdtC were produced from two plasmids and expressed in the same *E. coli*, a non-covalent CdtA-CdtC complex was formed in the absence of CdtB (Deng and Hansen, 2003). This CdtA-CdtC complex after association with HeLa cells (30 min, 4°C) was able to bind subsequently added CdtB, leading to cell killing within 72 h. Moreover, HeLa cells pretreated for 30 minutes at 37°C with the CdtA-CdtC complex were reported to become resistant to the cell killing induced by high concentrations of subsequently added HdCDT holotoxin (*H. ducreyi* culture supernatant). Surprisingly, however, such treated

cells turned out not to be resistant to the cell distention induced by lower amounts of the holotoxin (Deng and Hansen, 2003). This observation suggests that the cellular intoxication was not completely prevented.

Another recent piece of work considerably advanced our knowledge about binding of the CjCDT subunits to the HeLa cell surface (Lee *et al*., 2003). By competitive experiments, Lee and coworkers demonstrated that both the CdtA and CdtC subunits, but not CdtB, could bind specifically to the HeLa cell surface as detected by an enzyme-linked immunosorbent assay on living cells (CELISA). In unlabeled form, these subunits were able to reduce the binding of the corresponding biotinylated subunit. Interestingly, they could also compete with each other, suggesting that CdtA and CdtC were binding to the same receptor on the cell surface (Lee *et al*., 2003). Indeed, these two subunits of the CjCDT share about 40% sequence similarity with each other. McSweeney and Dreyfus recently noted the same binding competition between the CdtA and CdtC subunits of the EcCDT-II (McSweeney and Dreyfus, 2004).

Lee and coworkers (Lee *et al*., 2003), in addition, produced in-frame deletion mutants of CdtA and CdtC from which 43 and 22 amino acids, respectively, were deleted. Since the CdtA subunit from different bacteria shows several regions of homology, the authors chose to delete one of these regions from CjCdtA (amino acids 126–168). The amino acids 115–136 were deleted from the CjCdtC subunit because they were located in the region of highest homology between CdtCs from different bacteria. Both these mutant subunits in biotinylated form were still able to bind HeLa cells. Moreover, each mutant subunit was able to compete with the binding of both wild-type subunits, as well as that of the holotoxin. The binding of the CDT holotoxin decreased nearly fivefold in the presence of the CdtA mutant subunit and almost threefold in the presence of the CdtC mutant subunit. These interesting findings suggest that the deleted regions in each Cdt subunit do not play any significant role in cell surface recognition. On the other hand, the mutant subunits were unable to interact with CdtB and/or each other to reconstitute an active holotoxin, indicating that the deleted regions were instead critical for effective holotoxin formation (Lee *et al*., 2003).

### *Receptor for CDT*

No specific receptor for any CDT subunit has yet been identified, although receptor studies are currently going on in several laboratories. We recently demonstrated by immunofluorescence microscopy for the first time the direct binding of a CDT to the HeLa cell surface, using GST-labeled or fluorescein-conjugated HdCDT (Figure 23.3). Interestingly, we also found that extraction of membrane cholesterol with methyl-β-cyclodextrin (mβCD) before adding the toxin prevented cell surface binding of HdCDT and consequent intoxication (Guerra *et al*., 2005). Thus, it is likely that the receptor, at least for this variant of CDT, will be found among components enriched in the membrane microdomains known as lipid rafts, whose integrity is disrupted by drugs that extract cholesterol. It is also interesting to note that we were unable to demonstrate any binding of HdCDT to BalbC 3T3 fibroblasts, which have been previously reported to resist intoxication by CDT (Figure 23.3 and Cortes-Bratti *et al*., 1999). Apparently, the reason for their complete resistance is the lack of a functional CDT receptor, and this information might be helpful in future receptor studies.

### *Internalization of CDT and transport of CdtB via the Golgi complex and the ER*

The internalization process occurring after CDT binding to the cell surface has been studied only for the HdCDT. This toxin was reported to enter HEp-2/HeLa cells by endocytosis via clathrin-coated pits (Cortes-Bratti *et al*., 2000). The cellular intoxication was completely inhibited at conditions that block the fusion of early endo-

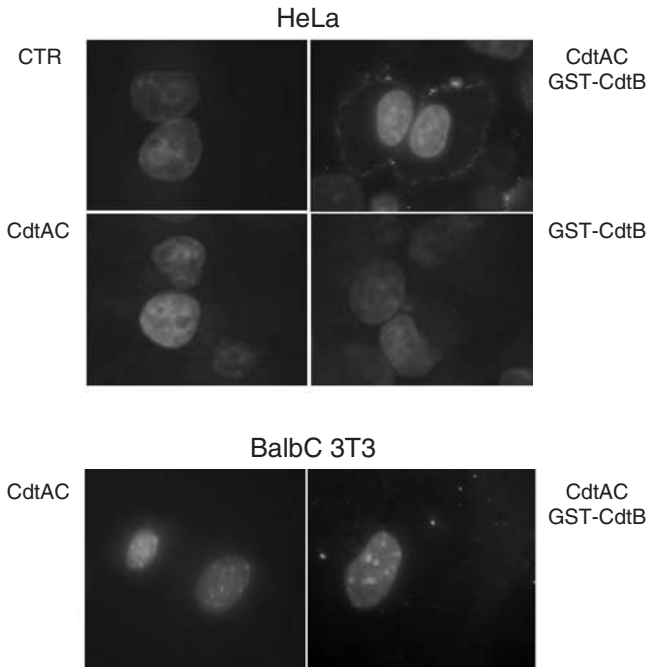



**FIGURE 23.3** Binding of CDT on the plasma membrane of sensitive cells. HeLa and BalbC 3T3 cells were left untreated (CTR) or exposed to the following subunit combinations: (i) CdtA+CdtC; (ii) GST-CdtB alone; (iii) CdtA+CdtC+GST-CdtB for 20 min on ice (the final concentration of each subunit was 0.2μg/μl). CDT binding was assessed by immunofluorescence staining, using a FITC-conjugated, anti-GST antibody. Nuclei were counterstained with Hoechst 33258.

somes with downstream compartments. Likewise, treatment of cells with agents that disrupt the Golgi complex (Brefeldin A, BFA, and Ilimaquinone) blocked the intoxication, suggesting that the toxin required transport in vesicles at least to the Golgi complex before its activity could be expressed (Cortes-Bratti *et al*., 2000). However, since BFA can affect also the transit through endosomes (van Dam *et al*., 2002), we have recently extended these observations by utilizing MDCK cells for which the Golgi complex, but not the endosomal compartment, has been shown to be BFA resistant (Hunziker *et al*., 1991). BFA did not induce any significant change in the HdCDT-induced intoxication of MDCK cells, in contrast to the results in HeLa cells, again supporting the involvement of the Golgi complex. Finally, a direct proof of CDT transit via the Golgi complex was recently obtained, since a CdtB genetically engineered to contain a sulfation site was shown to become sulphated, a reaction known to take place only in the Golgi complex (Guerra *et al*., 2005).

Several bacterial protein toxins have been shown to undergo retrograde transport from the Golgi complex to the ER. To clarify this aspect for HdCDT, we added also three N-glycosylation sites at the C-terminal of the CdtB subunit and were able to demonstrate its glycosylation. This finding indicated for the first time that HdCDT is also transported to the ER, the exclusive site for N-glycosylation reactions (Guerra *et al*., 2005).

***How does the CdtB subunit reach the nucleus?***

Toxins that are retrogradely transported to the ER and act on cytosolic targets, such as the ETA, ricin, and cholera toxin, are known to enter the cytosol only from the ER. The proposed mechanism for their transmembrane translocation involves the ER-associated degradation (ERAD) pathway (Simpson *et al*., 1999; Teter and Holmes, 2002), known to translocate misfolded secretory and ER membrane proteins into the cytosol for proteolytic degradation (Hampton, 2002). Several lines of evidence suggest that the ERAD pathway, using the Sec61p translocon, is essential for mediating cytosolic translocation of toxins that act in the cytosol, such as ricin and Shiga toxins. Both CHO (Teter and Holmes, 2002) and *S. cerevisiae* (Simpson *et al*., 1999) cells defective in various aspects of the ERAD pathway are resistant to ricin intoxication. Furthermore, sulfated and glycosylated ricin can be co-precipitated with anti-Sec61p–specific antibodies (Wesche *et al*., 1999). Also, cholera toxin is associated with and requires a functional Sec61p complex to be translocated from microsomes derived from porcine pancreas (Schmitz *et al*., 2000). In the case of the CDTs, there are two major options for reaching the molecular target: (i) CDT is translocated from the ER to the cytosol, perhaps as the toxins mentioned above, and then it enters the nucleus via nuclear pores or other transport mechanisms, or (ii) CDTs are directly translocated from the ER to the nucleus without any passage through the cytosolic compartment. Several groups are currently working on this interesting issue, as discussed below.

*Nuclear localization signals*

CdtB contains no known conventional nuclear localization signal (NLS). However, microinjection experiments in HeLa cells showed that a 76 amino acid stretch in the amino-terminal region (residues 48–124) in the AaCdtB constitutes an atypical NLS (Nishikubo *et al*., 2003). After microinjection, His-tagged CdtB-GFP entered the nucleus in 3–4 h. A lack of effect of leptomycin B on the speed of nuclear entry suggested that the relatively slow entry of the fusion protein was not due to CRM1-dependent nuclear export of the protein. An *in vitro* transport assay demonstrated that the nuclear localization of CdtB was mediated via active transport requiring ATP and physiological temperature. Moreover, cells treated extracellularly with a holotoxin containing mutant CdtB, with an 11-amino acid truncation in the identified NLS, were unaffected. This observation suggested that the identified amino-terminal NLS may be functional for nuclear localization of the toxin also when mammalian cells are naturally intoxicated with the holotoxin added extracellularly (Nishikubo *et al*., 2003). Subsequently, however, two NLS sequences, designated as NLS1 and NLS2, were identified in the carboxy-terminal region of the EcCdtB-II. Cell cycle arrest and nuclear localization were impaired in cells treated with CDT containing EcCdtB-II-ΔNLS mutants, while the *in vitro* DNase activity and the cell surface binding of the mutant holotoxins were not affected. Interestingly, fluorescence microscopy analysis showed a diffuse cytoplasmic distribution of the mutant CdtB in cells exposed to a CDT holotoxin containing the EcCdtB-II-ΔNLS2 subunit, while the EcCdtB-II-ΔNLS1 subunit localized preferentially in a perinuclear region upon internalization (McSweeney and Dreyfus, 2004).

*ERAD and Derlin-1 pathways are not required*

These interesting studies still do not tell us whether in natural intoxication the nuclear entry of CdtB takes place from the cytosol or directly from the ER. Therefore, we have recently studied HdCDT intoxication in mutant cell lines, defective in the only two ER-to-cytosol translocation machineries characterized so far—the ERAD and the Derlin-1 dependent pathways.

To assess whether the ERAD pathway is necessary for efficient translocation and intoxication of target cells by HdCDT, we used a set of Chinese hamster ovary (CHO) cell clones, which had been selected for resistance to ricin, ETA, and cholera toxins, due to an altered ERAD system (Teter and Holmes, 2002; Teter *et al*., 2003). These mutant cell lines are subgrouped in two classes: (i) one clone presenting a deficient translocation of proteins from the ER to the cytosol (CHO 16D), and (ii) two clones presenting an enhanced cytosolic degradation of retro-translocated proteins (CHO 23G and CHO 24D). Using the minimal toxin concentration able to induce cell cycle arrest in the parental CHO cells, we found that all three CHO mutant cell lines were equally sensitive to HdCDT intoxication as the parental cells, whether assessed by cell cycle arrest after 24 h or by a colony assay after two weeks (Guerra *et al*., 2005).

It has been shown recently that retro-translocation into the cytosol of a subset of proteins involves a human homologue of the yeast Der1 protein, named Derlin-1 (Lilley and Ploegh, 2004; Ye *et al*., 2004). To assess whether HdCDT internalization is Derlin-1 dependent, we investigated the sensitivity to HdCDT of HeLa cells expressing Derlin-$1^{GFP}$, which acts in a dominant negative manner, blocking the transport of proteins from the ER to the cytosol (Lilley and Ploegh, 2004). Interestingly, HeLa cells expressing the dominant negative Derlin-$1^{GFP}$ were found to be equally sensitive to HdCDT as the control cell lines were (Guerra *et al*., 2005).

Based on these observations, we were able to conclude that the trafficking of HdCDT at least does not involve any of these two ER-to-cytosol translocation pathways.

The data collected so far demonstrate that HdCDT and other toxins such as ricin, ETA, and cholera toxin follow a similar internalization pathway down to the ER. However, a major difference was observed when the requirement for the ER-associated degradation (ERAD) pathway was tested, since our results with HdCDT suggest that this toxin either does not need to transit to the cytosol before entering the nucleus, or alternatively that its translocation to the cytosol occurs via a different pathway. Since we also excluded a putative exit of HdCDT via the other known ER-cytosol translocation pathway (Lilley and Ploegh, 2004; Ye *et al*., 2004), we favor the hypothesis that HdCDT is transferred directly from the ER to the nucleus without passage through the cytosol.

As already mentioned, Mc Sweeney and Dreyfus (McSweeney and Dreyfus, 2004) noted that deletion of each of the C-terminal NLSs from the *E. coli* CdtB-II subunit produced a differential localization of the active toxin subunit. Cells intoxicated with a holotoxin containing the EcCdtB-II-ΔNLS1 displayed a perinuclear distribution of the active component, consistent with its trapping in the late endosome and/or ER compartment. On the other hand, a diffuse cytoplasmic staining was observed in cells exposed to the EcCdtB-II-ΔNLS2-containing toxin. Thus, the NLS1 might be crucial for ER-to-nucleus transport. An interesting possibility is that the NLS2 may act as scavenger motif to retrieve CdtB molecules that have escaped to the cytosol, similar to the suggested ER retention function of the KDEL sequence in cholera toxin (Lencer and Tsai, 2003). Also, the findings that the nuclear transport of wild-type AaCdtB required ATP and physiological temperature and was relatively slow (Nishikubo *et al*., 2003) are fully compatible with the hypothesis that CdtB is transported directly from the ER to the nucleus. It is more difficult, however, to reconcile the observations that the NLSs in the Aa-CdtB and Ec-CdtB-II, respectively, are located in entirely different parts of the CdtB molecules.

In conclusion, we hypothesize that the CdtB nuclear translocation requires an atypical mechanism, which has still not been characterized, but involves a direct translocation from the ER to the nucleus. Very recently, a new reticular network has been identified and designated as nucleoplasmic reticulum (Echevarria *et al*., 2003). This organelle is described as a fine, branching intranuclear network that is continuous with the nuclear envelope and the ER. A high degree of complexity of the ER network is further demonstrated by the fact that specific ER molecules can be found on phagosomes upon phagocytosis (Desjardins, 2003). Interaction between phagosomes and the ER might also explain how the *Salmonella typhi* CdtB (StCdtB), expressed only upon bacterial internalization and in the absence of CdtA and CdtC, could gain access to the nuclear compartment within 3–4 h after cellular infection (Haghjoo and Galan, 2004).

In a recent paper, Shenker and colleagues questioned whether the relevant activity of the CDT family of toxins is a DNase-like activity, and they suggested that the cytotoxic effects might be triggered upon toxin-induced transmembrane signaling from the cell surface (Shenker *et al*., 2004; Thelestam and Frisan, 2004). However, the studies cited previously demonstrate beyond any doubt that at least the active CdtB subunit has to be endocytosed and undergo intracellular transport to the nucleus before it can damage cells. Nothing has yet been reported regarding the cellular localizations and fates of the other two toxin subunits.

Figure 23.4 summarizes the internalization pathway of CDTs.

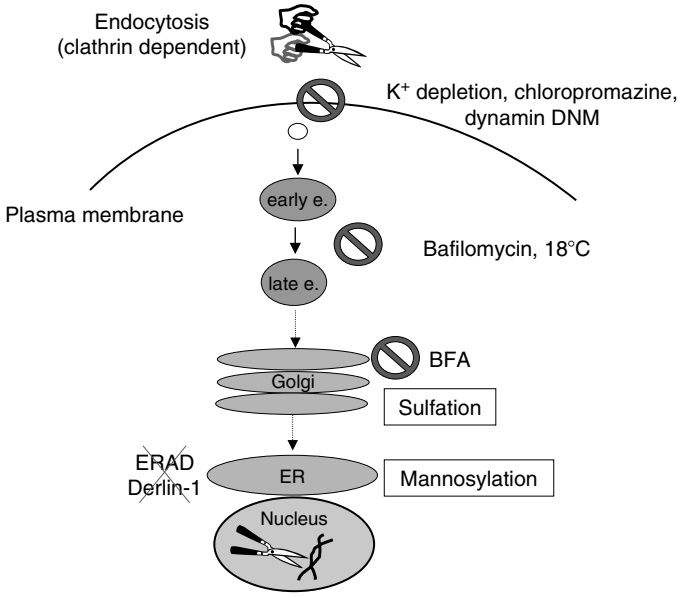



**FIGURE 23.4** CDT internalization pathway. CDTs are internalized via clathrin-dependent endocytosis into early and late endosomes. The CdtB subunit further transits to the Golgi complex (as assessed by sulfation experiments), and is then retrogradely transported to the endoplasmic reticulum (ER), as assessed by N-linked glycosylation. Further translocation from the ER does not require the ER-associated degradation (ERAD) pathway and is Derlin-1 independent.
The symbol Ø indicates that intoxication was abolished by the use of specific inhibitors or treatments.
Dynamin DNM: dynamin dominant negative mutant. BFA: Brefeldin A.

## Molecular mode of action of CDT

The next few sections will deal with the effect of CDTs on DNA, as well as the many cellular responses evoked by the CDT-induced DNA damage.

### *CdtB is a DNase*

On close analysis, all CdtBs turn out to have structural and functional homology to mammalian DNase I. The first description of position-specific homology between the CdtB subunit from EcCDT-II and mammalian DNase I was published by Elwell and Dreyfus (Elwell and Dreyfus, 2000). The homology pattern was found at specific residues that are involved in enzyme catalysis (Glu86, His154, Asp229, His261), DNA binding (Arg123, Asn194), and metal ion binding (Glu62, Asp192, Asp260). EcCdtB-II also contains a pentapeptide sequence (aa 259-263: Ser-Asp-His-Tyr-Pro) found in all DNase I enzymes. Shortly after, similar conserved residues were described for the *Campylobacter jejuni* CdtB (CjCdtB), and in fact identified in all known CdtBs (Lara-Tejero and Galan, 2000). The crystal structure of HdCdtB has been recently solved, and it shows the characteristic fold of the DNase I family: a central 12-stranded β-sandwich packed between outer α-helices and loops on each side of the sandwich (Nesic *et al.*, 2004).

The position-specific homology is associated with functional activity, since crude EcCDT-II preparations have DNase activity, as detected by *in vitro* digestion of the coiled pGEM-7zf plasmid (Elwell and Dreyfus, 2000). Transfection of HeLa cells with CjCdtB was shown to induce a slowly appearing nuclear fragmentation and a marked chromatin disruption (Lara-Tejero and Galan, 2000). Microinjected CjCdtB was also able to produce changes in the chromatin and enlargement of the nucleus. Both the DNase activity and the cytotoxicity were abolished by point mutations of conserved residues required for catalysis or for magnesium binding. Thus, the catalytic DNase activity is crucial for the cytotoxic activity of CDTs (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000).

### *CDT induces DNA double-strand breaks similar to ionizing radiation*

We recently provided direct evidence that intoxication of HeLa cells with the HdCDT holotoxin induces DNA double-strand breaks (DSBs) similar to IR (Figure 23.5a) (Frisan *et al.*, 2003). Moreover, mutation in the HdCdtB position 273, required for magnesium binding, completely abolished this effect (Figure 23.5a), as well as the cytotoxic responses. This first demonstration of CDT-induced DSBs in naturally intoxicated mammalian cells was made with a sensitive pulsed-field gel electrophoresis (PFGE) method. It contradicts an earlier report showing that EcCDT did not induce DNA strand breaks, as measured with the alkaline single cell gel electrophoresis (Comet) assay (Sert *et al.*, 1999). However, our findings agree with those of Hassane and coworkers who previously observed that yeast cells transfected with CjCdtB exhibited DSBs, also detectable by PFGE (Hassane *et al.*, 2001). Furthermore, this effect on DNA is fully consistent with the DNase activity of CdtB and the widely reported observations that CDT-treated cells exhibit cell cycle arrest and/or undergo apoptosis.

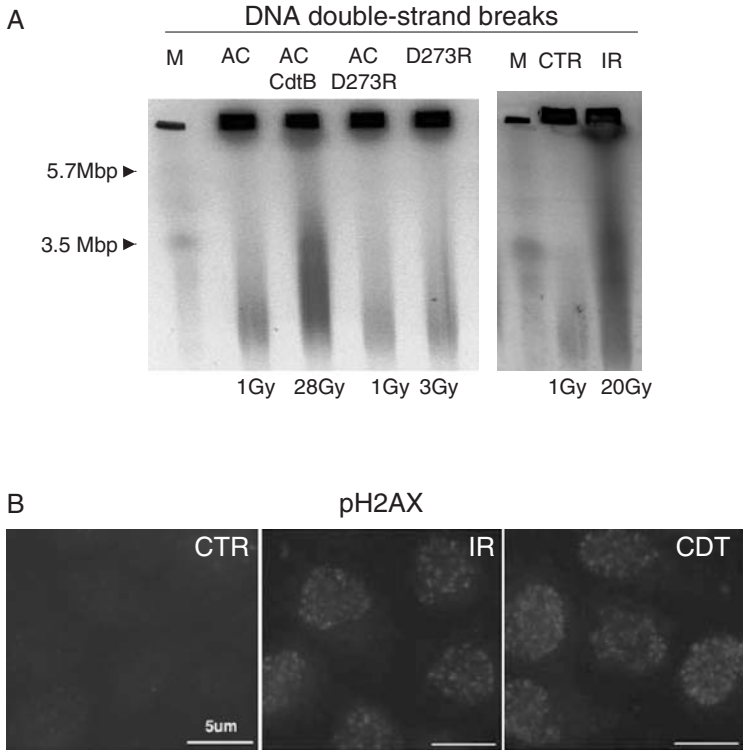



**FIGURE 23.5** CDT-induced DNA double-strand breaks (DSBs) and H2AX phosphorylation. A) Twenty-five thousand HeLa cells per well were cultivated in 12-well plates in complete medium containing 4,000Bq [methyl-$^{14}$C]-thymidine for 48h. The cells were then washed 3 times in PBS and chased for 2h in complete medium, and subsequently treated for 8h with: (i) CdtA+CdtC (negative control); (ii) HdCDT containing wild-type CdtB (at the concentration of 20µg/ml); (iii) HdCDT containing the mutated CdtBD273R (at the concentration of 20µg/ml); (iv) CdtBD273R alone (20µg/ml). Cells were processed for pulsed-field gel electrophoresis analysis. As control for DNA DSBs analysis, HeLa cells were left untreated or irradiated (20Gy, IR) and immediately processed for pulsed-field gel electrophoresis analysis.
M: molecular weight marker. Gy: equivalent radiation dose. **B)** HeLa cells were either incubated with medium alone (CTR), irradiated (20Gy, IR), or treated with HdCDT (2µg/ml, CDT) for 4h. Cells were fixed and stained with anti-phospho-H2AX specific rabbit serum, followed by FITC swine anti-rabbit serum.

### *CDT-induced cellular checkpoint responses*

We will first outline the cellular effects known to be elicited by DNA damaging agents in general and then present an overview of the cellular changes occurring specifically in response to various CDTs.

*Cellular effects of DNA damaging agents in general*

It is well known that cells exposed to agents causing DNA damage activate so-called "checkpoint responses" that arrest the cell cycle until the DNA damage has been repaired. These checkpoint responses can block cells in the G1, S, or G2 phases of the cell cycle (Elledge, 1996; Hartwell and Weinert, 1989). The protein kinase Ataxia telangiectasia mutated (ATM) and its homologue ATM and Rad3 related (ATR) play a central role in sensing DNA damage. ATM is activated in response to DNA DSBs induced by IR, and can in turn trigger all the different checkpoints. (ATR activation is mainly induced by other DNA-damaging agents, such as UV irradiation [Rotman and Shiloh, 1999]). Arrest in G1 is mediated by the tumor suppressor gene p53, which is stabilized in an ATM-dependent manner via phosphorylation on serine 20 by the chk2 protein kinase (Chehab *et al.*, 2000; Siciliano *et al.*, 1997). The G2 arrest depends on a loss of activation of the cdc2 complex. Normally, cdc2 activation is achieved via dephosphorylation at Thr-14 and Tyr-15 by the Cdc25C phosphatase (Jackman and Pines, 1997). The ATM-dependent protein kinases chk1 and chk2, activated in response to DNA damage, are able to inactivate Cdc25C (Matsuoka *et al.*, 1998; Sanchez *et al.*, 1997), and this leads to accumulation of the inactive phosphorylated cdc2 and arrest of cells in the G2 phase of the cell cycle.

*CDTs activate sensors of DNA damage*

We have demonstrated that HdCDT induces phosphorylation of the nuclear sensor of DNA damage, histone H2AX, as early as 1 h after intoxication (Figure 23.5b). Moreover, the DNA repair complex Mre11 was relocalized in the nuclei of HeLa cells with kinetics similar to those observed upon exposure to IR (Li *et al.*, 2002). Also, the CjCDT was reported to induce Rad50 foci in the nuclei of the CjCDT-treated primary human fetal fibroblasts (IMR-90 cells) (Hassane *et al.*, 2003). Thus, DNA-damage–associated molecules are activated as an early response to the DSBs induced by CDTs.

*CDT-induced cell cycle arrest*

Even before the toxin was known to damage DNA, cells exposed to various CDTs had been reported to accumulate the phosphorylated (inactive) form of cdc2, leading to arrest in the G2/M phase of the cell cycle (Comayras *et al.*, 1997; Cortes-Bratti *et al.*, 1999; Peres *et al.*, 1997; Whitehouse *et al.*, 1998). The inactive cdc2/cyclin B complex resulting after intoxication with

EcCDT-III could be reactivated *in vitro* with recombinant Cdc25C (Sert *et al*., 1999). Furthermore, overexpression of Cdc25B or Cdc25C could override the G2 arrest induced by this toxin, causing progression to a mitosis, which was abnormal, however (Escalas *et al*., 2000). Both results suggested that CDTs do not target specifically cdc2 but rather some upstream component, leading to inactivation of Cdc25C and consequent lack of cdc2 dephosphorylation as a secondary effect.

The cellular checkpoint responses induced by HdCDT were then shown to closely resemble those induced by IR. It also became clear that the CDT-induced cell cycle arrest is not limited to the G2/M phase, but that the checkpoint responses depend on the cell type (Cortes-Bratti *et al*., 2001b), as summarized in Table 23.2. In human fibroblasts, both IR and HdCDT treatment induced ATM-dependent early activation of the p53 gene and the cyclin-dependent kinase inhibitor p21, which correlated with arrest in G1 (Cortes-Bratti *et al*., 2001b). In HeLa cells, both treatments induced chk2 kinase activation, accumulation of phosphorylated cdc2, and G2 arrest (Cortes-Bratti *et al*., 2001b). The checkpoint responses were already detectable 4 h post-intoxication.

*CDT-induced apoptosis*

In contrast to fibroblasts and epithelial cells, human lymphoblastoid cell lines treated with HdCDT did not arrest in either G1 or G2 at the toxin concentrations used. Instead, they rapidly underwent apoptosis, a phenomenon that was not further studied in these cells (Cortes-Bratti *et al*., 2001b). Instead, CDT-induced apoptotic cell death has been investigated in some detail in human T cells and T cell lines. Treatment of activated human T cells with the AaCDT (purified B subunit or recombinant holotoxin) was found to induce DNA fragmentation in 72–96h, and FACS analysis showed reduction in cell size and increased nuclear condensation (Shenker *et al*., 2001). Mitochondrial changes were evident as a decrease in transmembrane potential and an elevation of reactive oxygen species, and the caspases 8, 9, and 3 were activated after the G2 arrest. Overexpression of Bcl-2 decreased the CDT-induced apoptosis without inhibiting the G2 arrest (Shenker *et al*., 2001). A more recent report on AaCDT-induced death of human peripheral T lymphocytes and the Jurkat and MOLT-4 cell lines, however, suggested that AaCDT has the ability to induce human T-cell apoptosis through activation of the caspases 2 and 7 (Ohara *et al*., 2004). Thus, the exact

**TABLE 23.2** Mammalian cell sensitive to CDT[a]

| Cell name | Cell type | G1 arrest | G2 arrest | Apoptosis | Species |
|---|---|---|---|---|---|
| HeLa | Epithelial | – | + | – | Human |
| HEp2 | Epithelial | – | + | – | Human |
| Caco2 | Epithelial | – | + | – | Human |
| Ca9-22 | Epithelial | – | + | – | Human |
| CHO | Epithelial | + | + | – | Hamster |
| C3H7An | Epithelial | nd | nd | nd | Mouse |
| | [b]Fibroblasts | + | + | – | Human |
| Don | Fibroblast | – | + | – | Hamster |
| Cos-7 | Fibroblast | – | + | + | Monkey |
| COS-1 | Fibroblast | – | + | – | Monkey |
| Vero | Fibroblast | nd | nd | nd | Monkey |
| | [b]CD4+ T lymphocytes | – | + | – | Human |
| | [b]CD8+ T lymphocytes | – | + | – | Human |
| Jurkat | T lymphocyte | – | + | + | Human |
| MOLT-4 | T lymphocyte | – | + | + | Human |
| BL41 | B lymphocyte | – | ±[c] | + | Human |
| JAC-B2 | B lymphocyte | – | ±[c] | + | Human |
| SN-B1 | B lymphocyte | – | ±[c] | + | Human |
| HS-72 | B cell hybridoma | – | + | + | Mouse |
| | [b]Macrophages | nd | nd | nd | Human |
| | [b]Dendritic cells | – | – | + | Human |
| HaCaT | Keratinocyte | – | + | – | Human |
| | [b]Keratinocytes | – | + | – | Human |
| | [b]Endothelial cells | + | + | – | Human |
| | [b]Periodontal ligament cells | + | + | – | Human |

[a]: most commonly reported CDT-sensitive cells

[b]: primary cell cultures

[c]: partial G2 arrest was detected in some experiments

molecular events on the pathway to the CDT-induced apoptotic cell death have not yet been fully elucidated, and they can be expected to depend on the particular type of target cell.

Figure 23.6 summarizes the checkpoint responses activated upon CDT intoxication.

### *CDT-induced Rho activation and stress response*

*Morphological effect of CDTs*

As already mentioned briefly, the most conspicuous morphological effect of CDT on cells growing as adherent monolayer is the cell distention leading within 72 h to a three- to fivefold increase of the cell size including a proportional nuclear enlargement. Along with this slowly developing distention, the actin cytoskeleton is strongly promoted, mainly noted as the appearance of prominent actin stress fibers (Figure 23.1). The stress fiber promotion has been reported for E. coli CDT (EcCDT-II) in CHO cells (Aragon *et al*., 1997), for HdCDT in HEp-2/HeLa cells, in Don hamster lung fibroblasts and human foreskin fibroblasts (Cortes-Bratti *et al*., 1999; Frisan *et al*., 2003), and recently also in HeLa cells treated with CDT from *Helicobacter cinaedi* (Taylor *et al*., 2003). After several days of toxin exposure, adherent cells begin to round up, show membrane blebbing in some cases, and then deteriorate completely. In contrast to fibroblasts and epithelial cells, T and B lymphocytes and dendritic cells do not distend but rather become rapidly apoptotic and fragmented after exposure to CDT (Cortes-Bratti *et al*., 2001b; Li *et al*., 2002; Shenker et al., 2004).

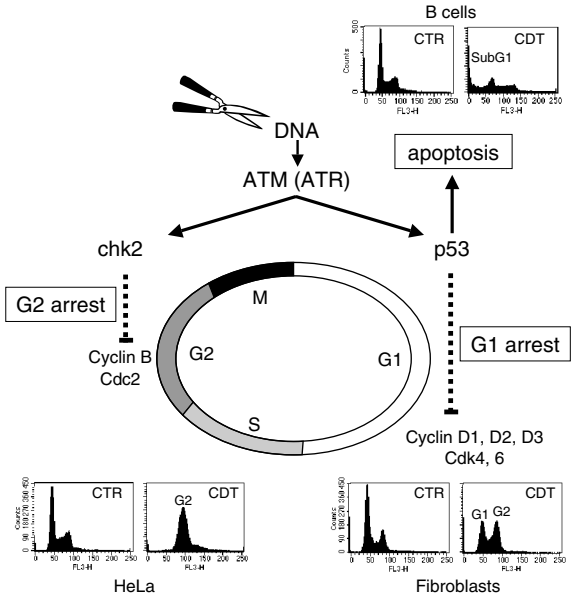



**FIGURE 23.6** Summary of the checkpoint responses activated upon CDT-induced DNA damage. The protein kinase ATM is activated upon CDT-induced DNA double-strand breaks. As a consequence of the DNA damage checkpoint responses, primary fibroblasts are arrested both in the G1 and G2 phases of the cell cycle, HeLa cells are exclusively arrested in G2, and B cells undergo apoptosis.

*CDT-induced stress fiber promotion depends on ATM-mediated activation of RhoA*

The reason that actin stress fibers are strongly promoted in certain CDT-treated cells was not understood in the early experiments (Aragon *et al*., 1997), although the possible involvement of small GTPases controlling the actin cytoskeleton was hypothesized (Cortes-Bratti *et al*., 1999). Later on, it became feasible to investigate this aspect of CDT intoxication with new tools that allow specific assay of the activated small GTPases Rho, Rac, and CDC42 (Benard *et al*., 1999; Ren *et al*., 1999). Thus, we could show that the HdCDT-activated stress fiber promotion in fibroblasts and HeLa cells depends on activation of RhoA, but not Rac or CDC42. This was observed both directly as activation of RhoA upon intoxication and as an inhibition of the stress fiber formation in CDT-treated cells transfected with a dominant negative mutant of RhoA (Frisan *et al*., 2003).

Interestingly, stress fiber promotion and cell distention were observed also in irradiated cells. However, the increased size of CDT-treated or irradiated cells was not connected to the stress fiber formation, since dominant negative RhoA inhibited the formation of stress fibers without affecting the distention of the treated cells (Frisan *et al*., 2003). The distention phenomenon instead appears to depend on activation of PI3-kinase and its downstream effector mTOR, whereas this enzyme had no clear effect on induction of stress fibers (Frisan *et al*., 2003). The activation of RhoA upon intoxication or irradiation was found to be ATM-dependent, thereby connecting the stress fiber promotion to the damaging action on DNA. Moreover, toxin-treated cells expressing a dominant negative form of RhoA detached and consequently died faster than cells expressing a functional RhoA (Frisan *et al*., 2003). Thus, the activation of RhoA was associated with prolonged cell survival, and it seems to represent an attempt of intoxicated cells to maintain cell adherence in order to stay alive while some time is given for DNA repair. The ATM-dependent activation of RhoA constitutes a previously unknown type of cell survival response to induction of DNA DSBs, which seems to occur regardless of the agent inducing the DSBs.

### *CDT-induced late cellular responses*

Only a limited number of studies have been devoted to responses occurring as secondary effects relatively late in cellular intoxication with CDTs. However, one study showed that each of the purified recombinant subunits

of AaCDT was individually able to induce production of the cytokines IL-1β, IL-6, and IL-8, but not TNFα, IL-12, or granulocyte-macrophage colony stimulating factor in human peripheral blood mononuclear cells (PBMC) as measured after 20 h incubation (Akifusa *et al*., 2001). CdtC was the most potent subunit, as 40 ng/ml CdtC was able to stimulate the production of nanogram quantities of IL-8. CdtB alone had only minimal cytokine stimulating activity, but it appeared to synergize with CdtA and CdtC to promote PBMC cytokine synthesis, and this synergy was most marked in inducing IFNγ production (Akifusa *et al*., 2001). The molecular mechanisms behind this effect are not clear, and the relation to DNA damage was not studied.

The effects of *Haemophilus ducreyi* and some of its antigens, such as HdCDT, were recently studied on human monocyte-derived dendritic cells (DCs), macrophages, and CD4+ T cells (Xu *et al*., 2004). While the whole bacteria induced antigen-presenting cells (APCs) to secrete TNF-α, IL-6, IL-8, and IL-12, the isolated toxin hampered both the induction of cytokine production by APCs and their ability to elicit T cell activation. DCs were shown to become slowly apoptotic, consistent with previous findings (Li *et al*., 2002). Thus, HdCDT is likely to inhibit the local immune responses in chancroid, besides delaying the healing of chancroid ulcers through the cytotoxic activity.

Both the AaCDT and HdCDT were recently reported to have a very interesting effect on periodontal connective tissue cells (Belibasakis *et al*., 2005). *A. actinomycetemcomitans* was shown by RT-PCR and protein determinations to induce the expression of Receptor Activator of NF-κB Ligand (RANKL) in human gingival fibroblasts and periodontal ligament cells. CDT was crucial for this effect as shown with (i) a CDT knockout mutant of the bacterium and (ii) a preparation where the CDT activity was neutralized with antibodies. Moreover, purified HdCDT (which has > 95% homology with the AaCDT) had the same RANKL-inducing effect. RANKL is known to induce osteoclast differentiation and activation (Teitelbaum and Ross, 2003). The authors speculate that CDT in this way might be indirectly involved in the pathological bone resorption process during localized aggressive periodontitis (Belibasakis *et al*., 2005).

### *CDTs act on several cell types*

As already discussed, CDTs act on various cell lines, and the outcomes differ depending on the cell type (Table 23.2). The most commonly reported end-points in CDT studies are cell cycle arrest and "cytotoxicity" based on morphological criteria. Cells reported until now to be sensitive to CDTs are summarized in Table 23.2. HEp-2, HeLa, CHO, Vero, and Don fibroblasts (Cortes-Bratti *et al*., 2001a; Pickett and Whitehouse, 1999), as well as human embryonic intestinal epithelial cells (INT407) (Hickey *et al*., 2000), a human colon carcinoma cell line (Caco-2), and a human keratinocyte cell line (HaCat) (Cortes-Bratti *et al*., 1999) are sensitive to CDTs. Human $CD4^+$ and $CD8^+$ T cells are highly sensitive to the AaCDT (Shenker *et al*., 1999), which also inhibits proliferation of the murine B-cell hybridoma cell line HS-72 (Sato *et al*., 2002), as well as periodontal ligament cells and gingival fibroblasts from humans (Belibasakis *et al*., 2002) and a human gingival squamous carcinoma cell line (Ca9-22) (Yamamoto *et al*., 2004). HdCDT affects normal human endothelial cells (HUVEC) (Svensson *et al*., 2002), foreskin keratinocytes and fibroblasts, embryonic lung fibroblasts, human B cells, and dendritic cells (Figures 23.6 and 23.7) (Cortes-Bratti *et al*.,

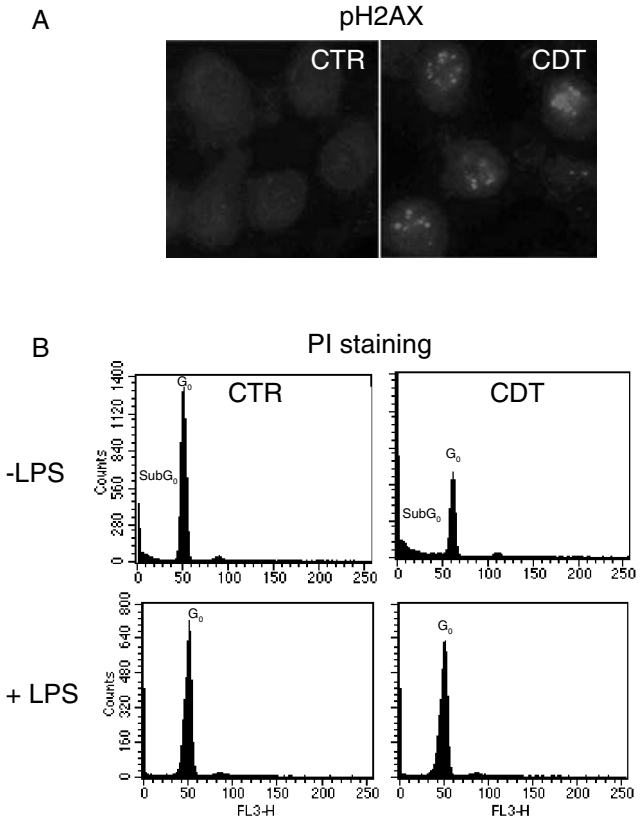



**FIGURE 23.7** CDT induces DNA damage and cell death in non-proliferating dendritic cells (DCs). **A)** Five days immature DCs were incubated with HdCDT (2μg/ml) for 5h and stained with histone H2AX phospho-specific rabbit serum (pH2AX). **B)** Effect on LPS-treated DCs. Five days immature DCs were incubated with or without LPS (1μg/ml) for 24h. Cells were further treated with HdCDT (2μg/ml) or left untreated for 48h. Cell cycle distribution was assessed by DNA staining with propidium iodide (PI) and flow cytometry (PI staining). The $G_0$ peak was arbitrarily set on the mean fluorescence intensity value of 50.

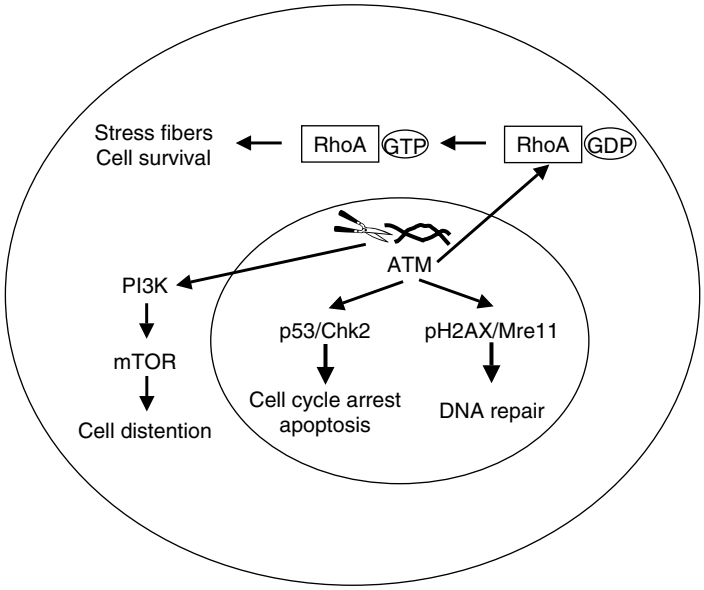



**FIGURE 23.8** Summary of the cellular responses activated by the CDT-induced DNA damage. CDT intoxication induces: (i) nuclear responses, leading to cell cycle arrest/apoptosis and activation of DNA repair complex; (ii) activation of cytosolic signaling pathways, important for prolonging cell survival.

2001b; Li *et al*., 2002). Primary human fetal fibroblasts (IMR-90) (Hassane *et al*., 2003), Cos-7 cells (McSweeney and Dreyfus, 2004), COS-1 (Sato *et al*., 2002), Jurkat, and MOLT-4 cells (Ohara *et al*., 2004) are also sensitive to CDT intoxication.

*CDTs act on non-proliferating cells*

It is noteworthy that CDTs attack also non-proliferating cells. Utilizing the focus formation assays of DNA repair enzymes, it was possible to establish that human dendritic cells (DCs) (Li *et al*., 2002), as well as serum-starved (i.e., non-proliferating) primary human fetal fibroblasts (Hassane *et al*., 2003), are sensitive to CDT. Phosphorylation of H2AX was detected in 50% to 60% of DCs exposed to HdCDT for 5h (Li *et al*., 2002; and Figure 23.7a). Cell cycle analysis shows that untreated DCs were in $G_0$ phase of the cell cycle, while a marked reduction of the cells in $G_0$ phase and a parallel increase of the sub$G_0$ population was observed in the HdCDT-treated DCs (Li *et al*., 2002; and Figure 23.7b). These findings contradict previous notions that cells need to pass through the S phase in order to become intoxicated (Alby *et al*., 2001; Lara-Tejero and Galan, 2000; Sert *et al*., 1999).

Interestingly, the effect of HdCDT on DCs was dependent on their stage of differentiation (Li *et al*., 2002), since LPS-treated DCs were resistant to HdCDT-induced cell death (Figure 23.7b). It is possible that upon DC maturation, the repertoire of surface molecules is modified in such a way that HdCDT is no longer able to bind and be internalized. This hypothesis is supported by the lack of DNA damaging activity observed in LPS-treated DCs exposed to HdCDT. Indeed, we could not demonstrate relocalization of the Mre11 complex or stabilization of the p53 protein in these cells, and H2AX phosphorylation was observed only in a small proportion of cells (Li *et al*., 2002).

In conclusion, most cultured cells that have been tested appear to be sensitive to CDT. The only cells so far reported to resist CDTs are the Y-1 adrenal cells and 3T3 fibroblasts of mouse origin (Cope *et al*., 1997; Cortes-Bratti *et al*., 1999; Johnson and Lior, 1988b). Since these cells were not able to bind HdCDT at all (Figure 23.3), the most probable reason for their resistance would be the lack of a cell surface receptor needed for binding of the toxin.

Figure 23.8 summarizes the intracellular responses activated by the CDT-induced DNA damage.

## CDTs AND DISEASE

Since CDTs are potent toxins produced by pathogenic bacteria, they have to be regarded as potential virulence factors. CDTs are detected in an increasing number of clinical isolates, but only a few experiments with CDTs in animal model systems have been reported.

### Occurrence of CDTs in clinical isolates

Several studies on *E. coli* isolates have been performed, especially concerning the possible association of CDT with enteropathogenic (EPEC) serotypes. The general conclusion from these studies is that EcCDT is produced in some, but not all, EPEC serotypes (Ansaruzzaman *et al*., 2000; Ghilardi *et al*., 2001; Janka *et al*., 2003; Okeke *et al*., 2000; Pandey *et al*., 2003). For example, only 1/200 children in Brazil (0.5%) with

acute diarrhea were infected with *cdt*-positive EPEC, while 1% of the non-diarrheic controls harbored *cdt*-positive isolates (Marques *et al*., 2003). CDT has been identified in urosepsis *E. coli* isolates (Johnson and Stell, 2000), in diarrhea isolates from dogs (Starcic *et al*., 2002) and from neonatal piglets (da Silva and da Silva Leite, 2002), in extraintestinal human and animal urinary tract infection (UTI) and non-UTI strains (Toth *et al*., 2003), and in 83% of bovine isolates of necrotoxigenic *E. coli* (Mainil *et al*., 2003).

Pickett and coworkers recently reported how the presence of *cdt* genes was related to a number of other virulence genes of *E. coli* (Pickett *et al*., 2004). Their study of a collection of 20 strains revealed that the CDT producers can be divided into three general groups with distinct differences in CDT type and in their complement of virulence-associated genes. Thus, *cdt* genes were found in: (i) a subset of uropathogenic *E. coli* strains; (ii) extraintestinal isolates of uncertain clonal identity; and (iii) in certain strains with characteristics in common with Shiga toxin–producing *E. coli* and EPEC (Pickett *et al*., 2004). CDT and the *cdt* genes were also identified in 17 out of 340 non-O157 Shiga toxin–producing *E. coli* (STEC) of serotypes that were all *eae*-negative (Bielaszewska *et al*., 2004). Among these *eae*-negative STEC, *cdt* was proposed to be disease associated, since it was significantly more frequent in isolates from patients with hemolytic uremic syndrome (3 of 7) and in isolates from patients with diarrhea (14 of 138) than in isolates from asymptomatic carriers (0 of 65).

The production of CDT has been detected in a few isolates of *Shigella dysenteriae*, *S. boydii*, and *S. sonnei* (Okuda *et al*., 1995). In contrast, almost all investigated *Campylobacter jejuni* and *C. coli* strains have been reported to produce CDT or to possess the *cdt* genes. For instance, the *cdtB* gene was detected in all investigated isolates from chicken carcasses, i.e., the primary source of *C. jejuni* and *C. coli* in human infections (Eyigor *et al*., 1999a; Eyigor *et al*., 1999b). *In vitro* assay showed that the *C. jejuni* isolates produced CDT at high toxin titers, whereas *C. coli* produced little or no toxin. Likewise, 100/101 *C. jejuni* and 7/10 *C. coli* isolates from Danish broilers had the *cdt* genes. Again the *C. coli* strains produced lower amounts of the toxin (Bang *et al*., 2001). CDT production was also demonstrated from *C. fetus*, and PCR experiments suggested the presence of *cdtB* sequences in other species of *Campylobacter* (Pickett *et al*., 1996). A study on several virulence genes, including the *cdt* cluster in *C. jejuni* and *C. coli* isolates from Danish pigs and cattle, demonstrated a high prevalence (83–95%) of *cdt* genes and CDT production (Bang *et al*., 2003). More recently, the same authors (Bang *et al*., 2004) reported that 90–97% of 117 *C. jejuni* isolates from Danish turkeys produced CDT, as detected by cytotoxicity in three different cell lines.

In contrast to the situation in *Campylobacter* spp, the *cdt* genes and CDT activity are not present in all species of *Helicobacter*. The three genes have been identified in *H. hepaticus* (Young *et al*., 2000a), and the *cdtB* gene was detected in human clinical isolates of *H. pullorum* (Young *et al*., 2000b) and in canine isolates of *H. flexispira* (Kostia *et al*., 2003), but no CDT homology was found in *H. pylori* (Chien *et al*., 2000). Interestingly, CDT was recently reported as the first putative virulence factor present in *H. cinaedi*, which is the most commonly reported enterohepatic helicobacter in humans (Taylor *et al*., 2003). The *cdtB* gene detected by PCR, as well as CDT production detected by cytotoxicity, was present in all 11 investigated isolates, 10 of which were clinical isolates.

Out of 50 periodontitis strains of *A. actinomycetemcomitans*, 86% had the *cdt* genes and expressed cytotoxic activity (Ahmed *et al*., 2001). A majority (39/40) of clinical isolates from Brazil, Kenya, Japan, and Sweden were also found to harbor the *cdt* genes and produce the AaCDT (Fabris *et al*., 2002). Although there was some variation in toxin production among the strains, no clear relationship between CDT activity and periodontal status could be found. CDT production and occurrence of the *cdtB* gene were also reported in 73 strains of periodontopathogenic bacteria (Yamano *et al*., 2003), and CDT activity was found in 40/45 tested *A. actinomyctemcomitans* strains. The remaining 28 strains of other bacteria did not have CDT. In another recent study *A. actinomycetemcomitans* was detected in 106/146 subgingival plaque samples isolated from periodontitis patients, and among these only 13 sites were positive for the *cdt* genotype (Tan *et al*., 2002). However, 10 of these 13 positive sites were obtained from patients diagnosed with aggressive periodontitis, and the authors speculated that the cytotoxicity and immunosuppression by CDT may contribute to the pathogenesis of aggressive periodontitis (Tan *et al*., 2002). The RANKL-inducing activity of AaCDT, reported more recently (Belibasakis *et al*., 2005), might also contribute in the pathogenesis of this disease.

Finally, 89% of a group of isolates of *H. ducreyi* from different parts of the world showed CDT activity on HEp-2 cells (Purvén *et al*., 1995). A later study identified the *cdt* genes and CDT production in 83% of 29 isolates of *H. ducreyi* from chancroid (Ahmed *et al*., 2001). Likewise, 87% of 45 strains of *H. ducreyi,* found in still more recent chancroid isolates from various parts of the world, produced the toxin (Kulkarni *et al*., 2003).

In conclusion, a majority of strains of *C. jejuni*, *A. actinomycetemcomitans*, and *H. ducreyi* appear to

Table 23.3 Summary of the CDT occurrence in clinical isolates

| Toxin | Bacteria/disease | Species infected |
|---|---|---|
| *E. coli* CDTs | EPEC | Human |
| | Urosepsis *E. coli* | Human |
| | Diarrhea isolates | Dog |
| | Diarrhea isolates | Pig |
| | UTI strains | Human/animal |
| | Non-UTI strains | Human/animal |
| | Necrotoxigenic | Bovine |
| | STEC | Human |
| *Shigella* CDTs | Few isolates | |
| *Campylobacte*r CDTs | *C. jejuni* | Chicken/human |
| | *C. coli* | Chicken/human |
| | *C. fetus* | |
| *Helicobacter* CDTs | *H. pullorum* | Human |
| | *H. flexispira* | Human |
| | *H. cinaedi* | Human |
| AaCDT | Aggressive periodontitis | Human |
| HdCDT | Chancroid | Human |

produce the CDT, while the presence of the *cdt* genes in other bacteria is more variable. Table 23.3 summarizes the data reported in the previous paragraphs.

## Effects of CDTs *in vivo*

An *E. coli* strain expressing the *S. dysenteriae cdt* genes was able to induce watery diarrhea in the suckling mouse model, and the partially purified CDT also had some effect. In addition, the toxin caused a certain tissue damage in the descending colon of these mice (Okuda *et al*., 1997). When *cdtB* negative strains of *C. jejuni* were administered to severe combined immunodeficient mice, no difference in enteric colonization was observed. However, the CDT-deficient strains showed an impaired invasiveness into blood, spleen, and liver tissues as compared to the wild-type bacteria (Purdy *et al*., 2000), implying that CDT might have a role in the pathogenesis (invasion) of *C. jejuni*. A more recent study of a *C. jejuni cdtB* mutant in NF-κB deficient mice suggested that CDT may contribute to the pro-inflammatory response, as well as the ability of *C. jejuni* to escape immune surveillance (Fox *et al*., 2004). The *cdtB* mutant was able to colonize the NF-κB deficient 3X mice to the same extent as the wild-type bacteria, but produced significantly less gastritis (Fox *et al*., 2004), suggesting that CDT may be important for the pathogenic process taking place after the colonization. The authors speculate that CDT may target the cells of the immune system in the lamina propria, therefore influencing the host's ability to clear bacterial pathogens. Young and coworkers recently provided further evidence for a potential pro-inflammatory activity of CDTs (Young *et al*., 2004). Strains of *H. hepaticus* expressing a functional CDT caused severe colitis in a murine model of inflammatory bowel disease, using C57BL/6 interleukin 10 (IL-10) deficient mice. In contrast, the CDT-negative isogenic mutants were without effect (Young *et al*., 2004).

An isogenic *H. ducreyi cdtC* mutant was equally virulent as the parent strain when tested in the temperature-dependent rabbit model for experimental chancroid, despite the fact that it was not cytotoxic to HeLa cells and keratinocytes (Stevens *et al*., 1999). Isogenic *H. ducreyi cdtA* and *cdtB* mutants also proved to be as virulent as the wild-type strain with regard to lesion production in the same rabbit model (Lewis *et al*., 2001). In human volunteers, expression of CDT also was not required for pustule formation by *H. ducreyi*, although CDT might still be relevant for induction or persistence of the ulcer stage, which is not testable in humans (Young *et al*., 2001). The purified HdCDT holotoxin induced dose-dependent pathologic skin reactions in rabbits (Wising *et al*., 2002). High levels of neutralizing antibodies against CDT were detected in only 22% and 2% of patients with chancroid and periodontitis, respectively. Moreover, a majority of healthy individuals also had HdCDT antibodies, and thus such antibodies may not be specific markers for chancroid infection (Mbwana *et al*., 2003).

In conclusion, the fact that CDTs are produced by diverse human pathogens suggests that these toxins might contribute to the development of different diseases. However, there is currently no absolutely clear association between toxins and specific disease symptoms, and one major problem is the lack of suitable animal models for these types of studies. Conceivably, CDT could have a role in all instances where cell proliferation is required. Thus, CDTs produced by intestinal pathogens could possibly contribute to gastroenteritis by blocking the proliferation of crypt cells, although there are no studies yet on this particular aspect of the *E. coli*, *Campylobacter*, or *Shigella* CDTs. The HdCDT could contribute to the very slow healing of chancroid ulcers. Furthermore, immunosuppression could be one important general effect of CDTs, as suggested by the recent studies cited previously and by the *in vitro* effect of CDT on DCs, which have a central role for induction of both cellular and humoral-mediated immunoresponses (Li *et al*., 2002). Targeting DCs could represent a strategy developed by several pathogens in order to avoid or delay the onset of immunoresponses. In fact, the AaCDT was originally isolated as the "immunosuppressive factor" (ISF) of *A. actinomycetemcomitans*, and it might possibly play such a role in the pathogenesis of aggressive periodontitis and other infections caused by this bacterium

(Shenker *et al*., 1999). Finally, the recent evidence that AaCDT can induce expression of the osteoclast-activating factor RANKL in periodontal connective tissue cells further supports a role for CDT in localized aggressive periodontitis.

## CONCLUSION

During the 10 years after the first cloning of CDT, this tripartite toxin has generated an increasing interest, and the development has advanced rapidly from mysterious cell distending activity to well-known toxin with defined mode of action and now even crystallized. CDT turned out to be unique in that it is the only bacterial toxin known to target DNA as a primary action, subsequently activating a number of important cellular stress responses designed to save the attacked cell while it attempts to repair its DNA. Although we know today so much more than a decade ago, there are many more questions waiting for their answers. In addition, there is reason to believe that CDT, like many other bacterial protein toxins, can be developed into a useful tool in cell biology and biomedicine.

### Action of CDT on cells

The receptor(s) for CDT has not yet been identified. This issue is complicated by the tripartite CDT structure with two separate binding subunits, CdtA and CdtC, which might bind to separate receptor structures, or to the same (as suggested in Lee *et al*., 2003; McSweeney and Dreyfus, 2004). The sequence homologies between the CdtA and CdtC subunits from different bacteria are much lower than between the different CdtB subunits. Thus, it is even possible that the receptor(s) for CDTs of different origins will turn out to differ despite the fact that the active CdtB subunit in all CDTs has the same DNase activity. That there are lectin-like regions in CdtA and CdtC similar to those found in the binding subunit of ricin might point to a galactose-containing receptor, although Mao and DiRienzo (Mao and DiRienzo, 2002) failed to inhibit the binding of AaCdtA to CHO cells with various galactosides and mannosides. Besides the binding to cell surface receptor(s), the three subunits must be able to interact with each other to form the fully active holotoxin. Obviously, this interaction must depend on specific amino acid stretches, some of which have already been identified. It would also be of interest to combine subunits of CDTs from different bacteria to see whether they can fully complement each other.

It is clear that CDT has to be internalized in cells so that the CdtB subunit can be transferred to the nucleus. Upon microinjection, CdtB is able to enter the nucleus from the cytosol, but the molecular events of this transfer process are not known. Moreover, it is not yet clear how the CdtB subunit is translocated from the ER to the nucleus upon natural intoxication. A passage through the cytosol seems less likely, according to the most recent observations that neither the ERAD-pathway nor Derlin-1 is necessary for the translocation. The cellular fates of CdtA and CdtC, after fulfilling their putative roles to facilitate entry of CdtB, have also not been clarified. Another unresolved issue is whether CdtC, if it really proves to be cytotoxic by itself, has an entirely different mode of action than CdtB.

### CDTs and disease

It will be interesting to see when CDTs will be more clearly demonstrated to cause or at least to contribute to the symptoms in infections caused by the bacteria producing them. Now that the mode of action of CDT is basically known, this aspect will probably become more attractive for study, even if the lack of really good animal models is still a problem. In any case, CDTs are likely to affect the immune response as well as other situations in which cell proliferation is needed, such as the healing of chancroid or periodontic ulcers. Another potentially important aspect is that CDTs, being genotoxic, might possibly constitute a contributing factor in long-term cancer development. Since CDT is produced by so many common pathogens, this is a rather disturbing perspective, which has not yet been studied at all. On the other hand, the action of CDT on DNA makes it a potentially good candidate for an anti-tumor agent.

### CDTs as tools in cell biology

Bacterial toxins have been extremely useful in the study of different aspects of cell biology. So far, CDTs are the only bacterial protein toxins known to induce a subtle DNA damage. In the field of cell and tumor biology, regulation of the cell cycle is currently one of the major issues, and CDT will probably be utilized as a tool in such studies. Toxins have also been very helpful in the study of endocytosis and intracellular transport in general. Studies with Shiga toxin showed for the first time that a molecule can be transported from the cell surface to the Golgi complex and the ER (Sandvig and Van Deurs, 2000). The ER appears to have a central role in regulation and execution of processes involving intracellular trafficking, some of which are still unknown. In this context, elucidation of the mechanism by which CdtB is translocated into the nucleus becomes an important issue.

Finally, the study of CDT has already led to the discovery of a novel cellular signaling pathway, transmitting survival signals from damaged DNA to the small GTPase RhoA (Frisan *et al*., 2003). CDT will be useful in the continued efforts to clarify all the molecular details from DNA damage to RhoA activation, as well as from the latter event to the downstream components involved in protecting the cells from immediate death.

## ACKNOWLEDGMENTS

Work from the authors' laboratory has been supported by the Swedish Research Council (grants # 05969 and 15012) and the Karolinska Institutet. T.F. was supported by the Swedish Cancer Society, the Swedish Society for Medical Research, and the Swedish Research Council.

## REFERENCES

Ahmed, H.J., Svensson, L.A., Cope, L.D., Latimer, J.L., Hansen, E.J., Ahlman, K., Bayat-Turk, J., Klamer, D. and Lagergard, T. (2001). Prevalence of cdtABC genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. *J. Med. Microbiol*., **50,** 860–864.

Akifusa, S., Poole, S., Lewthwaite, J., Henderson, B. and Nair, S.P. (2001). Recombinant *Actinobacillus actinomycetemcomitans* cytolethal distending toxin proteins are required to interact to inhibit human cell cycle progression and to stimulate human leukocyte cytokine synthesis. *Infect. Immun*., **69,** 5925–5930.

Alby, F., Mazars, R., de Rycke, J., Guillou, E., Baldin, V., Darbon, J.-M. and Ducommun, B. (2001). Study of the cytholethal distending toxin (CDT)-activated cell cycle checkpoint. Involvement of the CHK2 kinase. *FEBS Letters*, **491,** 261–265.

Ansaruzzaman, M., Albert, M.J., Nahar, S., Byun, R., Katouli, M., Kuhn, I. and Mollby, R. (2000). Clonal groups of enteropathogenic *Escherichia coli* isolated in case-control studies of diarrhea in Bangladesh. *J. Med. Microbiol*., **49,** 177–185.

Aragon, V., Chao, K. and Dreyfus, L.A. (1997). Effect of cytolethal distending toxin on F-actin assembly and cell division in Chinese hamster ovary cells. *Infect. Immun*., **65,** 3774–3780.

Bang, D.D., Borck, B., Nielsen, E.M., Scheutz, F., Pedersen, K. and Madsen, M. (2004). Detection of seven virulence and toxin genes of *Campylobacter jejuni* isolates from Danish turkeys by PCR and cytolethal distending toxin production of the isolates. *J. Food Prot*., **67,** 2171–2177.

Bang, D.D., Nielsen, E.M., Scheutz, F., Pedersen, K., Handberg, K. and Madsen, M. (2003). PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *J. Appl. Microbiol*., **94,** 1003–1014.

Bang, D.D., Scheutz, F., Ahrens, P., Pedersen, K., Blom, J. and Madsen, M. (2001). Prevalence of cytolethal distending toxin (cdt) genes and CDT production in *Campylobacter spp*. isolated from Danish broilers. *J. Med. Microbiol*., **50,** 1087–1094.

Belibasakis, G., Johansson, A., Wang, Y., Chen, C., Kalfas, S. and Lerner, U.H. (2005). The cytolethal distending toxin induces receptor activator of NF-κB ligand expression in human gingival fibroblasts and periodontal ligament cells. *Infect. Immun.*, **73,** 342–351.

Belibasakis, G., Johansson, A., Wang, Y., Claesson, R., Chen, C., Asikainen, S. and Kalfas, S. (2002). Inhibited proliferation of human periodontal ligament cells and gingival fibroblasts by *Actinobacillus actinomycetemcomitans*: involvement of the cytolethal distending toxin. *Eur. J. Oral. Sci*., **110,** 366–373.

Benard, V., Bohl, B.P. and Bokoch, G.M. (1999). Characterization of Rac and Cdc42 activation in chemoattractant-stimulated neutrophils using a novel assay for active GTPases. *J. Biol. Chem*., **274,** 13198–13204.

Bielaszewska, M., Fell, M., Greune, L., Prager, R., Fruth, A., Tschape, H., Schmidt, M.A. and Karch, H. (2004). Characterization of cytolethal distending toxin genes and expression in Shiga toxin-producing *Escherichia coli* strains of non-O157 serogroups. *Infect. Immun*., **72,** 1812–1816.

Chehab, N.H., Malikzay, A., Appel, M. and Halazonetis, T. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G1 by stabilizing p53. *Genes & Development*, **14,** 278–288.

Chien, C.C., Taylor, N.S., Ge, Z., Schauer, D.B., Young, V.B. and Fox, J.G. (2000). Identification of cdtB homologues and cytolethal distending toxin activity in enterohepatic *Helicobacter spp. J. Med. Microbiol*., **49,** 525–534.

Comayras, C., Tasca, C., Peres, S.Y., Ducommun, B., Oswald, E. and De Rycke, J. (1997). *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G2/M transition by preventing cdc2 protein kinase dephosphorylation and activation. *Infect. Immun*., **65,** 5088–5095.

Cope, L.D., Lumbley, S., Latimer, J.L., Stevens, M.K., Johnson, L.S., Purvén, M., Munson, R.S., Lagergård, T., Radolf, J. and Hansen, E.J. (1997). A diffusible cytotoxin of *Haemophilus ducreyi. PNAS*, **94,** 4056–4061.

Cortes-Bratti, X., Chaves-Olarte, E., Lagergård, T. and Thelestam, M. (1999). The cytolethal distending toxin from the chancroid bacterium *Haemophilus ducreyi* induces cell cycle arrest in the G2 phase. *J. Clin. Investig*., **103,** 107–115.

Cortes-Bratti, X., Chaves-Olarte, E., Lagergård, T. and Thelestam, M. (2000). Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi. Infect. Immun*., **68,** 6903–6911.

Cortes-Bratti, X., Frisan, T. and Thelestam, M. (2001a). The cytolethal distending toxins induce DNA damage and cell cycle arrest. *Toxicon*, **39,** 1729–1736.

Cortes-Bratti, X., Karlsson, C., Lagergård, T., Thelestam, M. and Frisan, T. (2001b). The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. *J. Biol. Chem*., **276,** 5296–5302.

da Silva, A.S. and da Silva Leite, D. (2002). Investigation of putative CDT gene in *Escherichia coli* isolates from pigs with diarrhea. *Vet. Microbiol*., **89,** 195–199.

Deng, K. and Hansen, E.J. (2003). A CdtA-CdtC complex can block killing of HeLa cells by *Haemophilus ducreyi* cytolethal distending toxin. *Infect. Immun*., **71,** 6633–6640.

Deng, K., Latimer, J.L., Lewis, D.A. and Hansen, E.J. (2001). Investigation of the interaction among the components of the cytolethal distending toxin of *Haemophilus ducreyi. Biochem. Biophys. Res. Commun*., **285,** 609–615.

Desjardins, M. (2003). ER-mediated phagocytosis: a new membrane for new functions. *Nat. Rev. Immunol*., **3,** 280–291.

Echevarria, W., Leite, M.F., Guerra, M.T., Zipfel, W.R. and Nathanson, M.H. (2003). Regulation of calcium signals in the nucleus by a nucleoplasmic reticulum. *Nat. Cell Biol*., **5,** 440–446.

Elledge, S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. *Science*, **274,** 1664–1672.

Elwell, C., Chao, K., Patel, K. and Dreyfus, L. (2001). *Escherichia coli* CdtB mediates cytolethal distending toxin cell cycle arrest. *Infect. Immun*., **69,** 3418–3422.

Elwell, C.A. and Dreyfus, L.A. (2000). DNAase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. *Mol. Microbiol*., **37,** 952–963.

Escalas, N., Davezac, N., De Rycke, J., Baldin, V., Mazars, R. and Ducommun, B. (2000). Study of the cytolethal distending toxin-induced cell cycle arrest in HeLa cells: involvement of the Cdc25 phosphatases. *Exp. Cell Res*., **257,** 206–212.

Eyigor, A., Dawson, K.A., Langlois, B.E. and Pickett, C.L. (1999a). Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter coli* isolates: detection and analysis by PCR. *J. Clin. Microbiol*., **37,** 1646–1650.

Eyigor, A., Dawson, K.A., Langlois, B.E. and Pickett, C.L. (1999b). Detection of cytolethal distending toxin activity and *cdt* genes in *Campylobacter spp*. isolated from chicken carcasses. *Appl. Environ. Microbiol*., **65,** 1501–1505.

Fabris, A.S., DiRienzo, J.M., Wikstrom, M. and Mayer, M.P. (2002). Detection of cytolethal distending toxin activity and cdt genes in *Actinobacillus actinomycetemcomitans* isolates from geographically diverse populations. *Oral Microbiol. Immunol*., **17,** 231–238.

Fox, J.G., Rogers, A.B., Whary, M.T., Ge, Z., Taylor, N.S., Xu, S., Horwitz, B.H. and Erdman, S.E. (2004). Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. *Infect. Immun*., **72,** 1116–1125.

Frisan, T., Cortes-Bratti, X., Chaves-Olarte, E., Stenerlöw, B. and Thelestam, M. (2003). The *Haemophilus ducreyi* cytolethal distending toxin induces DNA double-strand breaks and promotes ATM-dependent activation of RhoA. *Cell Microbiol*., **5,** 695–707.

Frisk, A., Lebens, M., Johansson, C., Ahmed, H., Svensson, L., Ahlman, K. and Lagergård, T. (2001). The role of different protein components from the *Haemophilus ducreyi* cytolethal distending toxin in the generation of cell toxicity. *Microb. Pathog*., **30,** 313–324.

Ghilardi, A.C., Gomes, T.A. and Trabulsi, L.R. (2001). Production of cytolethal distending toxin and other virulence characteristics of *Escherichia coli* strains of serogroup O86. *Mem. Inst. Oswaldo. Cruz*, **96,** 703–708.

Guerra, L., Teter, K., Lilley, B.N., Stenerlow, B., Holmes, R.K., Ploegh, H.L., Sandvig, K., Thelestam, M. and Frisan, T. (2005). Cellular internalization of cytolethal distending toxin: a new end to a known Pathway. *Cell Microbiol*. **7,** 921–934.

Haghjoo, E. and Galan, J.E. (2004). *Salmonella typhi* encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. *PNAS*, **101,** 4614–4619.

Hampton, R.Y. (2002). ER-associated degradation in protein quality control and cellular regulation. *Curr. Opin. Cell Biol*., **14,** 476–482.

Hartwell, L.H. and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science*, **246,** 629–634.

Hassane, D.C., Lee, R.B., Mendenhall, M.D. and Pickett, C.L. (2001). Cytolethal distending toxin demonstrates genotoxic activity in a yeast model. *Infect. Immun*., **69,** 5752–5759.

Hassane, D.C., Lee, R.B. and Pickett, C.L. (2003). *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. *Infect. Immun*., **71,** 541–545.

Hickey, T.E., McVeigh, A.L., Scott, D.A., Michielutti, R.E., Bixby, A., Carroll, S.A., Bourgeois, A.L. and Guerry, P. (2000). *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect. Immun*., **68,** 6535–6541.

Hunziker, W., Whitney, J.A. and Mellman, I. (1991). Selective inhibition of transcytosis by brefeldin A in MDCK cells. *Cell*, **67,** 617–627.

Jackman, M.R. and Pines, J.N. (1997). Cyclins and the G2/M transition. *Cancer Surv*., **29,** 47–73.

Janka, A., Bielaszewska, M., Dobrindt, U., Greune, L., Schmidt, M.A. and Karch, H. (2003). Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. *Infect. Immun*., **71,** 3634–3638.

Johnson, J.R. and Stell, A.L. (2000). Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J. Infect. Dis*., **181,** 261–272.

Johnson, W.M. and Lior, H. (1987a). Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. *FEMS Microbiology Letters*, **43,** 19–23.

Johnson, W.M. and Lior, H. (1987b). Production of Shiga toxin and a cytolethal distending toxin (CLDT) by serogroups of *Shigella* spp. *FEMS Microbiology Letters*, **48,** 235–238.

Johnson, W.M. and Lior, H. (1988a). A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter spp*. *Microb. Pathog*., **4,** 115–126.

Johnson, W.M. and Lior, H. (1988b). A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. *Microb. Pathog*., **4,** 103–113.

Kostia, S., Veijalainen, P., Hirvi, U. and Hanninen, M.L. (2003). Cytolethal distending toxin B gene (*cdtB*) homologues in taxa 2, 3, and 8 and in six canine isolates of *Helicobacter sp. flexispira*. *J. Med. Microbiol*., **52,** 103–108.

Kulkarni, K., Lewis, D.A. and Ison, C.A. (2003). Expression of the cytolethal distending toxin in a geographically diverse collection of *Haemophilus ducreyi* clinical isolates. *Sex Transm. Infect*., **79,** 294–297.

Lara-Tejero, M. and Galan, J.E. (2000). A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science*, **290,** 354–357.

Lara-Tejero, M. and Galan, J.E. (2001). CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect. Immun*., **69,** 4358–4365.

Lee, R.B., Hassane, D.C., Cottle, D.L. and Pickett, C.L. (2003). Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. *Infect. Immun*., **71,** 4883–4890.

Lencer, W.I. and Tsai, B. (2003). The intracellular voyage of cholera toxin: going retro. *Trends Biochem. Sci*., **28,** 639–645.

Lewis, D.A., Stevens, M.K., Latimer, J.L., Ward, C.K., Deng, K., Blick, R., Lumbley, S.R., Ison, C.A. and Hansen, E.J. (2001). Characterization of *Haemophilus ducreyi* cdtA, cdtB, and cdtC mutants in *in vitro* and *in vivo* systems. *Infect. Immun*., **69,** 5626–5634.

Li, L., Sharipo, A., Chaves-Olarte, E., Masucci, M.G., Levitsky, V., Thelestam, M. and Frisan, T. (2002). The *Haemophilus ducreyi* cytolethal distending toxin activates sensors of DNA damage and repair complexes in proliferating and non-proliferating cells. *Cell Microbiol*., **4,** 87–99.

Lilley, B.N. and Ploegh, H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature*, **429,** 834–840.

Mainil, J.G., Jacquemin, E. and Oswald, E. (2003). Prevalence and identity of cdt-related sequences in necrotoxigenic *Escherichia coli*. *Vet. Microbiol*., **94,** 159–165.

Mao, X. and DiRienzo, J.M. (2002). Functional studies of the recombinant subunits of a cytolethal distending holotoxin. *Cell Microbiol*., **4,** 245–255.

Marques, L.R., Tavechio, A.T., Abe, C.M. and Gomes, T.A. (2003). Search for cytolethal distending toxin production among fecal *Escherichia coli* isolates from Brazilian children with diarrhea and without diarrhea. *J. Clin. Microbiol*., **41,** 2206–2208.

Matsuoka, S., Huang, M. and Elledge, S.J. (1998). Linkage of ATM to cell cycle regulation by the chk2 protein kinase. *Science*, **282,** 1893–1897.

Mayer, M.P., Bueno, L., Hansen, E.J. and DiRienzo, J.M. (1999). Identification of a cytolethal distending toxin gene locus and

features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. *Infect. and Immun.*, **67,** 1227–1237.

Mbwana, J., Ahmed, H.J., Ahlman, K., Sundaeus, V., Dahlen, G., Lyamuya, E. and Lagergard, T. (2003). Specificity of antibodies directed against the cytolethal distending toxin of *Haemophilus ducreyi* in patients with chancroid. *Microb. Pathog.*, **35,** 133–137.

McSweeney, L.A. and Dreyfus, L.A. (2004). Nuclear localization of the *Escherichia coli* cytolethal distending toxin CdtB subunit. *Cell Microbiol.*, **6,** 447–458.

Nesic, D., Hsu, Y. and Stebbins, C.E. (2004). Assembly and function of a bacterial genotoxin. *Nature*, **429,** 429–433.

Nishikubo, S., Ohara, M., Ueno, Y., Ikura, M., Kurihara, H., Komatsuzawa, H., Oswald, E. and Sugai, M. (2003). An N-terminal segment of the active component of the bacterial genotoxin cytolethal distending toxin B (CDTB) directs CDTB into the nucleus. *J. Biol. Chem.*, **278,** 50671–50681.

Ohara, M., Hayashi, T., Kusunoki, Y., Miyauchi, M., Takata, T. and Sugai, M. (2004). Caspase-2 and caspase-7 are involved in cytolethal distending toxin-induced apoptosis in Jurkat and MOLT-4 T-cell lines. *Infect. Immun.*, **72,** 871–879.

Okeke, I.N., Lamikanra, A., Steinruck, H. and Kaper, J.B. (2000). Characterization of *Escherichia coli* strains from cases of childhood diarrhea in provincial southwestern Nigeria. *J. Clin. Microbiol.*, **38,** 7–12.

Okuda, J., Fukumoto, M., Takeda, Y. and Nishibuchi, M. (1997). Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdt* ABC genes of *Shigella dysenteriae*. *Infect. Immun.*, **65,** 428–433.

Okuda, J., Kurazono, H. and Takeda, Y. (1995). Distribution of the cytolethal distending toxin A gene (*cdt*A) among species of *Shigella* and *Vibrio* and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae*. *Microb. Pathog.*, **18,** 167–172.

Pandey, M., Khan, A., Das, S.C., Sarkar, B., Kahali, S., Chakraborty, S., Chattopadhyay, S., Yamasaki, S., Takeda, Y., Nair, G.B. and Ramamurthy, T. (2003). Association of cytolethal distending toxin locus cdtB with enteropathogenic *Escherichia coli* isolated from patients with acute diarrhea in Calcutta, India. *J. Clin. Microbiol.*, **41,** 5277–5281.

Peres, S.Y., Marches, O., Daigle, F., Nougayrede, J.P., Herault, F., Tasca, C., De Rycke, J. and Oswald, E. (1997). A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. *Mol. Microbiol.*, **24,** 1095–1107.

Pickett, C.L., Cottle, D.L., Pesci, E.C. and Bikah, G. (1994). Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infect. Immun.*, **62,** 1046–1051.

Pickett, C.L., Lee, R.B., Eyigor, A., Elitzur, B., Fox, E.M. and Strockbine, N.A. (2004). Patterns of variations in *Escherichia coli* strains that produce cytolethal distending toxin. *Infect. Immun.*, **72,** 684–690.

Pickett, C.L., Pesci, E.C., Cottle, D.L., Russell, G., Erdem, A.N. and Zeytin, H. (1996). Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. cdtB gene. *Infect. Immun.*, **64,** 2070–2078.

Pickett, C.L. and Whitehouse, C.A. (1999). The cytolethal distending toxin family. *Trends Microbiol.*, **7,** 292–297.

Purdy, D., Buswell, C.M., Hodgson, A.E., McAlpine, K., Henderson, I. and Leach, S.A. (2000). Characterization of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. *J. Med. Microbiol.*, **49,** 473–479.

Purvén, M., Falsen, E. and Lagergård, T. (1995). Cytotoxin production in 100 strains of *Haemophilus ducreyi* from different geographic locations. *FEMS Microbiology Letters*, **129,** 221–224.

Ren, X.D., Kiosses, W.B. and Schwartz, M.A. (1999). Regulation of small GTP-binding protein rho by cell adhesion and the cytoskeleton. *EMBO*, **18,** 578–585.

Rotman, G. and Shiloh, Y. (1999). ATM: a mediator of multiple responses to genotoxic stress. *Oncogene*, **18,** 6135–6144.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science*, **277,** 1497–1501.

Sandvig, K. and Van Deurs, B. (2000). Entry of Ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. *EMBO J*, **19,** 5943–5950.

Sato, T., Koseki, T., Yamato, K., Saiki, K., Konishi, K., Yoshikawa, M., Ishikawa, I. and Nishihara, T. (2002). p53-independent expression of p21(CIP1/WAF1) in plasmacytic cells during G(2) cell cycle arrest induced by Actinobacillus actinomycetemcomitans cytolethal distending toxin. *Infect. Immun.*, **70,** 528–534.

Schmitz, A., Herrgen, H., Winkeler, A. and Herzog, V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. *J. Cell Biol.*, **148,** 1203–1212.

Scott, D.A. and Kaper, J.B. (1994). Cloning and sequencing of the genes encoding Escherichia coli cytolethal distending toxin. *Infect. Immun.*, **62**, 244–251.

Sert, V., Cans, C., Tasca, C., Bret-Bennis, L., Oswald, E., Ducommun, B. and De Rycke, J. (1999). The bacterial cytolethal distending toxin (CDT) triggers a G2 cell cycle checkpoint in mammalian cells without preliminary induction of DNA strand breaks. *Oncogene*, **18,** 6296–6304.

Shenker, B.J., Besack, D., McKay, T., Pankoski, L., Zekavat, A. and Demuth, D.R. (2004). *Actinobacillus actinomycetemcomitans* cytolethal distending toxin (Cdt): evidence that the holotoxin is composed of three subunits: CdtA, CdtB, and CdtC. *J. Immunol.*, **172,** 410–417.

Shenker, B.J., Hoffmaster, R.H., McKay, T.L. and Demuth, D.R. (2000). Expression of the cytolethal distending toxin (Cdt) operon in *Actinobacillus actinomycetemcomitans*: evidence that CdtB protein is responsible for G2 arrest of the cell cycle in human T cells. *J. Immunol.*, **165,** 2612–2618.

Shenker, B.J., Hoffmaster, R.H., Zekavat, A., Yamaguchi, N., Lally, E.T. and Demuth, D.R. (2001). Induction of apoptosis in human T cells by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin is a consequence of G2 arrest of the cell cycle. *J. Immunol.*, **167,** 435–441.

Shenker, B.J., McKay, T., Datar, S., Miller, M., Chowhan, R. and Demuth, D. (1999). *Actinobacillus actinomycetemcomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. *J. Immunol.*, **162,** 4773–4680.

Siciliano, J.D., Canman, C.E., Taya, Y., Sakaguchi, K., Appella, E. and Kastan, M.B. (1997). DNA damage induces phosphorylation of the amino terminus of p53. *Genes & Development*, **11,** 3471–3481.

Simpson, J.C., Roberts, L.M., Romisch, K., Davey, J., Wolf, D.H. and Lord, J.M. (1999). Ricin A chain utilizes the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast. *FEBS Lett.*, **459,** 80–84.

Starcic, M., Johnson, J.R., Stell, A.L., van der Goot, J., Hendriks, H.G., van Vorstenbosch, C., van Dijk, L. and Gaastra, W. (2002). Hemolytic *Escherichia coli* isolated from dogs with diarrhea have characteristics of both uropathogenic and necrotoxigenic strains. *Vet. Microbiol.*, **85,** 361–377.

Stevens, M.K., Latimer, J.L., Lumbley, S.R., Ward, C.H., Cope, L.D., Lagergård, T. and Hansen, E.J. (1999). Characterization of a

*Haemophilus ducreyi* mutant deficient in the production of the cytolethal distending toxin. *Infect. Immun.*, **67,** 3900–3908.

Sugai, M., Kawamoto, T., Peres, S.Y., Ueno, Y., Komatsuzawa, H., Fujiwara, T., Kurihara, H., Suginaka, H. and Oswald, E. (1998). The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. *Infect. Immun.*, **66,** 5008–5019.

Svensson, L.A., Henning, P. and Lagergard, T. (2002). The cytolethal distending toxin of *Haemophilus ducreyi* inhibits endothelial cell proliferation. *Infect. Immun.*, **70,** 2665–2669.

Tan, K.S., Song, K.P. and Ong, G. (2002). Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. *J. Periodontal. Res.*, **37,** 268–272.

Taylor, N.S., Ge, Z., Shen, Z., Dewhirst, F.E. and Fox, J.G. (2003). Cytolethal distending toxin: a potential virulence factor for *Helicobacter cinaedi*. *J. Infect. Dis.*, **188,** 1892–1897.

Teitelbaum, S.L. and Ross, F.P. (2003). Genetic regulation of osteoclast development and function. *Nat. Rev. Genet.*, **4,** 638–649.

Teter, K. and Holmes, R.K. (2002). Inhibition of endoplasmic reticulum-associated degradation in CHO cells resistant to cholera toxin, *Pseudomonas aeruginosa* exotoxin A, and ricin. *Infect. Immun.*, **70,** 6172–6179.

Teter, K., Jobling, M.G. and Holmes, R.K. (2003). A class of mutant CHO cells resistant to cholera toxin rapidly degrades the catalytic polypeptide of cholera toxin and exhibits increased endoplasmic reticulum-associated degradation. *Traffic*, **4,** 232–242.

Thelestam, M. and Frisan, T. (2004). *A. actinomycetemcomitans* cytolethal distending toxin. *J. Immunol.*, **172,** 5813; author reply 5813–5814.

Toth, I., Herault, F., Beutin, L. and Oswald, E. (2003). Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV). *J. Clin. Microbiol.*, **41,** 4285–4291.

van Dam, E.M., Ten Broeke, T., Jansen, K., Spijkers, P. and Stoorvogel, W. (2002). Endocytosed transferrin receptors recycle via distinct dynamin and phosphatidylinositol 3-kinase-dependent pathways. *J. Biol. Chem.*, **277,** 48876–48883.

Wesche, J., Rapak, A. and Olsnes, S. (1999). Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J. Biol. Chem.*, **274,** 34443–34449.

Whitehouse, C.A., Balbo, P.B., Pesci, E.C., Cottle, D.L., Mirabito, P.M. and Pickett, C.L. (1998). Campylobacter jejuni cytolethal distending toxin causes a G2-phase cell cycle block. *Infect. Immun.*, **66,** 1934–1940.

Wising, C., Svensson, L.A., Ahmed, H.J., Sundaeus, V., Ahlman, K., Jonsson, I.M., Molne, L. and Lagergard, T. (2002). Toxicity and immunogenicity of purified *Haemophilus ducreyi* cytolethal distending toxin in a rabbit model. *Microb. Pathog.*, **33,** 49–62.

Xu, T., Lundqvist, A., Ahmed, H.J., Eriksson, K., Yang, Y. and Lagergard, T. (2004). Interactions of *Haemophilus ducreyi* and purified cytolethal distending toxin with human monocyte-derived dendritic cells, macrophages, and CD4+ T cells. *Microbes Infect.*, **6,** 1171–1181.

Yamamoto, K., Tominaga, K., Sukedai, M., Okinaga, T., Iwanaga, K., Nishihara, T. and Fukuda, J. (2004). Delivery of cytolethal distending toxin B induces cell cycle arrest and apoptosis in gingival squamous cell carcinoma *in vitro*. *Eur. J. Oral. Sci.*, **112,** 445–451.

Yamano, R., Ohara, M., Nishikubo, S., Fujiwara, T., Kawamoto, T., Ueno, Y., Komatsuzawa, H., Okuda, K., Kurihara, H., Suginaka, H., Oswald, E., Tanne, K. and Sugai, M. (2003). Prevalence of cytolethal distending toxin production in periodontopathogenic bacteria. *J. Clin. Microbiol.*, **41,** 1391–1398.

Ye, Y., Shibata, Y., Yun, C., Ron, D. and Rapoport, T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature*, **429,** 841–847.

Young, R.S., Fortney, K.R., Gelfanova, V., Phillips, C.L., Katz, B.P., Hood, A.F., Latimer, J.L., Munson JR, R.S., Hansen, E.J. and Spinola, S.M. (2001). Expression of cytolethal distending toxin and hemolysin is not required for pustule formation by *Haemophilus ducreyi* in human volunteers. *Infect. Immun.*, **69,** 1938–1942.

Young, V.B., Chien, C.C., Knox, K.A., Taylor, N.S., Schauer, D.B. and Fox, J.G. (2000b). Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. *J. Infect. Dis.*, **182,** 620–623.

Young, V.B., Knox, K.A., Pratt, J.S., Cortez, J.S., Mansfield, L.S., Rogers, A.B., Fox, J.G. and Schauer, D.B. (2004). *In vitro* and *in vivo* characterization of *Helicobacter hepaticus* cytolethal distending toxin mutants. *Infect. Immun.*, **72,** 2521–2527.

Young, V.B., Knox, K.A. and Schauer, D.B. (2000a). Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. *Infect. Immun.*, **68,** 184–191.

CHAPTER

# 24

# *Helicobacter pylori* vacuolating toxin

*Mark S. McClain and Timothy L. Cover*

## INTRODUCTION

*Helicobacter pylori* is a Gram-negative, spiral-shaped bacterium that colonizes the human stomach (Warren and Marshall, 1983; Marshall and Warren, 1984; Dunn *et al*., 1997; Cover *et al*., 2001; Suerbaum and Michetti, 2002). It has been estimated that about 50% of the world's population is colonized by *H. pylori*, with this proportion reaching greater than 90% in certain parts of the world. Despite the fact that most hosts mount humoral and cellular immune responses against *H. pylori*, colonization commonly persists for decades in the absence of antimicrobial therapy (Everhart, 2000). Infection by *H. pylori* is associated with an increased risk for development of peptic ulcer disease, gastric adenocarcinoma, and gastric lymphoma (Dunn *et al*., 1997; Cover *et al*., 2001; Suerbaum and Michetti, 2002).

An exotoxin produced by *H. pylori*, VacA, was discovered in 1988 (Leunk *et al*., 1988). Substantial evidence indicates that the toxin plays a role as a virulence factor in the pathogenesis of *H. pylori*–induced gastric diseases. One of the most striking activities of this toxin is its capacity to induce formation of large cytoplasmic vacuoles in cultured epithelial cells (Leunk *et al*., 1988; Cover and Blaser, 1992). However, VacA also produces an assortment of other cellular effects. VacA contributes to the capacity of *H. pylori* to colonize the stomach in a murine model of infection. In addition, VacA interferes with the activation and proliferation of T lymphocytes, which might be a factor that enables *H. pylori* to resist clearance by host immune defenses. Many of the activities attributed to the toxin, including cell vacuolation, are associated with the capacity of the toxin to form anion-selective membrane channels. Current studies are aimed at understanding the molecular mechanisms by which the toxin interacts with cells to produce multiple effects. In addition to providing further insights into *H. pylori* pathogenesis, these studies have implications for diverse fields of cell biology, including the study of lipid rafts, endocytosis and intracellular trafficking, apoptosis, cell proliferation, signal transduction, and chloride channels.

*H. pylori* colonization of the stomach is a complex process that involves bacterial adaptation to the acidic gastric environment, motility, adhesion, and interactions with the gastric epithelium and with host immune defenses. The VacA toxin is one factor that *H. pylori* apparently utilizes to make the human stomach a hospitable niche (Montecucco *et al*., 1999; Atherton *et al*., 2001; Papini *et al*., 2001). This review discusses our current understanding of VacA, including its synthesis, genetic diversity, association with disease, activities, interaction with host cells, and structure.

## VacA SYNTHESIS AND GENETIC DIVERSITY

The VacA toxin is the product of a single chromosomal gene (*vacA*) that is present in all *H. pylori* strains (Cover *et al*., 1994; Phadnis *et al*., 1994; Schmitt and Haas, 1994; Telford *et al*., 1994). The gene is transcribed from its own promoter (Schmitt and Haas, 1994; Forsyth *et al*., 1998; Forsyth and Cover, 1999), and expression of *vacA* is regulated by a variety of environmental stresses that *H. pylori* might encounter in the human host. One study reported that transcription of *vacA* is increased in response to acidic pH via a mechanism requiring CsrA (carbon storage regulator A), an RNA binding protein involved in posttranscriptional regulation (Barnard

*et al*., 2004). Other studies have reported that *vacA* transcription is increased in response to the presence of cultured gastric epithelial cells (van Amsterdam *et al*., 2003) and in response to iron limitation (Szczebara *et al*., 1999; Merrell *et al*., 2003).

The *vacA* gene encodes for a ~140 kDa protein, which undergoes proteolytic processing at the amino terminus to remove a ~33 amino acid signal sequence and at the carboxy terminus to remove a ~50 kDa domain (Cover *et al*., 1994; Phadnis *et al*., 1994; Schmitt and Haas, 1994; Telford *et al*., 1994; Nguyen *et al*., 2001) (Figure 24.1A). The carboxy-terminal portion of this 50 kDa domain exhibits ~40% amino acid similarity to the IgAβ-core domain from the IgA-protease of *Neisseria gonorrhoeae* (Pohlner *et al*., 1987; Schmitt and Haas, 1994), a representative of the autotransporter family (type V secretion pathway) of secreted bacterial proteins (Henderson *et al*., 1998; Desvaux *et al*., 2004). This region of VacA also includes a carboxy-terminal amino acid motif ending in phenylalanine that is frequently found among outer membrane proteins of Gram-negative bacteria (Struyvé *et al*., 1991; Cover *et al*., 1994). Two cysteines, separated by 10 to 12 amino acids, are located near the C-terminus of the mature toxin, and a similar motif is found in several other members of the autotransporter family of secreted bacterial proteins (Yanagida *et al*., 1986; Pohlner *et al*., 1987; Gilmore *et al*., 1989; Poulsen *et al*., 1989; Carl *et al*., 1990; Benz and Schmidt, 1992; Cover *et al*., 1994). One study investigated the functional role of the carboxy-terminal portion of the VacA protoxin by fusing it to the cholera toxin B subunit and expressing this fusion protein in *E. coli* (Fischer *et al*., 2001). The CtxB-VacA fusion protein expressed in *E. coli* was localized to the outer membrane, and the CtxB domain was shown by protease digestion experiments to be exposed on the bacterial surface. This capacity of the VacA carboxy-terminal region to transport the cholera toxin B subunit provides evidence that the carboxy-terminal portion of VacA may serve as an autotransporter in *H. pylori*. In *H. pylori*, it remains to be determined whether the mature 88 kDa VacA toxin is cleaved from the autotransporter

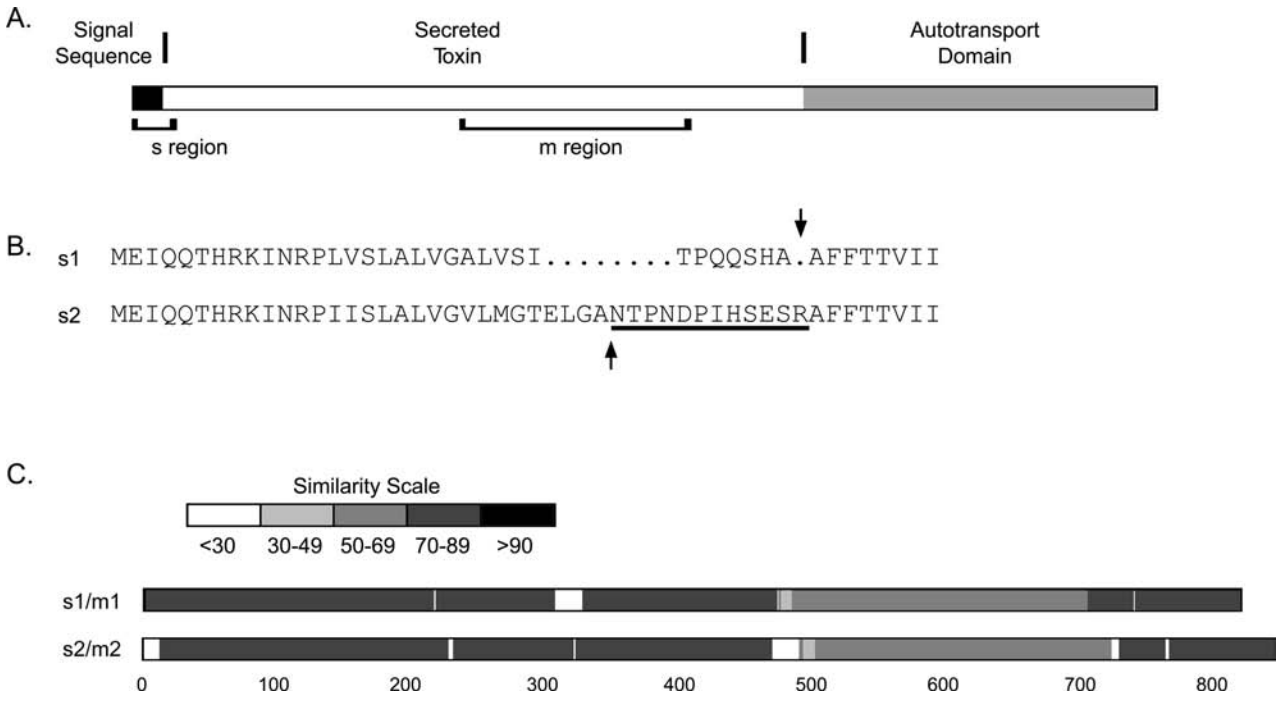



**FIGURE 24.1** VacA primary structure and amino acid diversity. A. The organization of the VacA protoxin is depicted. Proteolytic cleavage of an amino-terminal signal sequence and a carboxy-terminal autotransporter domain results in a mature, 88-kDa secreted toxin. Two regions of VacA diversity (s region and m region) are indicated. B. The "s" regions from prototypical s1 (*H. pylori* strain 60190, GenBank accession U05676) and s2 (*H. pylori* strain Tx30a, GenBank accession U29401) VacA toxins are shown. Arrows indicate the site at which the amino-terminal signal sequence is cleaved. The underlined residues correspond to a 12 amino acid extension located at the amino-terminus of s2-type VacA toxins. C. The amino acid sequences of prototypical s1-m1 (*H. pylori* strain 60190) and s2-m2 (*H. pylori* strain Tx30a) mature VacA toxins were aligned using the SIM local similarity program (Huang and Miller, 1991) and the similarity between the two sequences is depicted graphically (Duret *et al*., 1996). The numbering scheme represents amino acid positions. Several regions displaying less than 30% identity correspond to insertions or deletions in one of the two proteins.

domain by autoproteolysis or by the activity of an unidentified protease. A 33 kDa protein corresponding to the carboxy-terminus of the VacA autotransporter domain remains localized with the bacteria (Telford *et al*., 1994; Fischer *et al*., 2001), and a ~12 kDa fragment is secreted into the culture supernatant, suggesting that an additional proteolytic event removes this fragment (Bumann *et al*., 2002).

Following removal of the amino-terminal signal sequence and carboxy-terminal autotransporter domain, the mature 88 kDa VacA is secreted into the culture medium, though a significant fraction of the 88 kDa toxin may remain associated with the bacterial cell surface (Telford *et al*., 1994). The toxin that remains associated with the bacterium can be delivered to eukaryotic cells either via membrane blebs (Fiocca *et al*., 1999; Keenan *et al*., 2000) or through a contact-dependent mechanism (Ilver *et al*., 2004). The primary amino acid sequence of the mature VacA toxin resembles little else in GenBank. A BLAST search of the full-length VacA sequence reveals relatedness to three paralogous proteins from each of the two sequenced *H. pylori* genomes (Tomb *et al*., 1997; Alm *et al*., 1999) and a pair of proteins from *H. bilis* (Feng *et al*., 2002) (Figure 24.2). The similarity, however, is largely limited to the carboxy-terminal autotransporter domain of VacA (Figure 24.2). In addition, sequences have been found in two *H. acinonychis* isolates that exhibit 84% nucelotide identity to *vacA* from *H. pylori*, but these sequences contain numerous insertions, deletions, and translational stops (Dailidiene *et al*., 2004).

There is considerable variation among different *H. pylori* strains with respect to the levels of toxic activity found in broth culture supernatants (Leunk *et al*., 1988; Cover *et al*., 1993a; Atherton *et al*., 1995). Some strains appear to harbor inactive genes containing deletions or other mutations within the *vacA* open reading frame (Ito *et al*., 1998). Among strains that contain active genes, both the level of *vacA* transcription and the efficiency of VacA secretion may differ (Cover and Blaser, 1992; Cover *et al*., 1993a; Forsyth *et al*., 1998). However, differences in the quantity of secreted toxin do not wholly account for the differences in toxin activity observed in culture super-

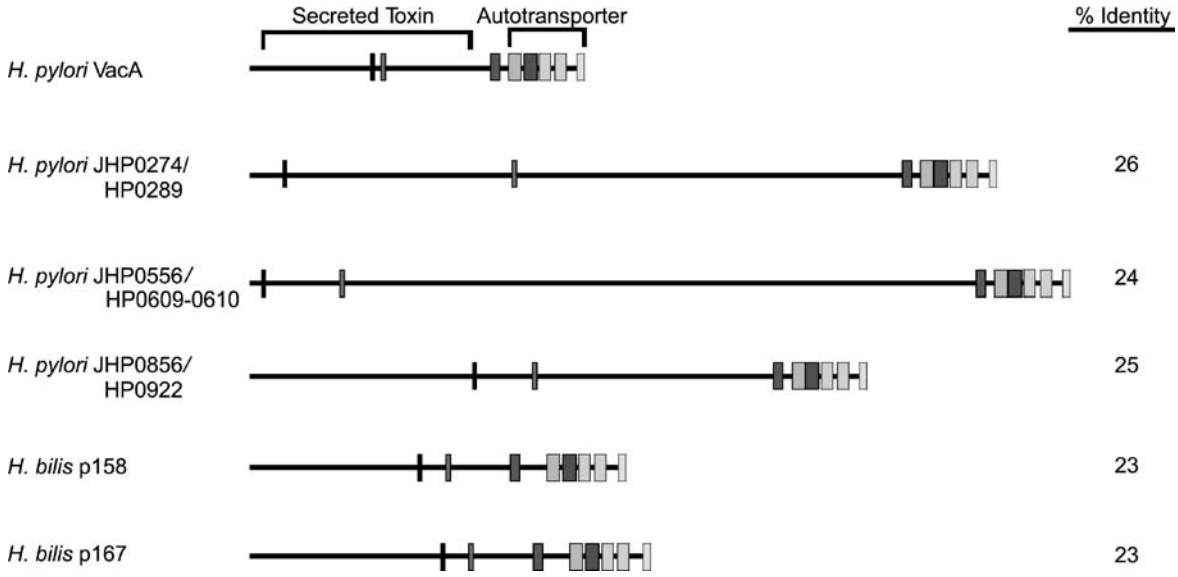



**FIGURE 24.2** VacA and related proteins. The amino acid sequence of the VacA protoxin from *H. pylori* strain 60190 was analyzed using the BLAST local alignment search tool (Altschul *et al*., 1990). Other than additional VacA sequences, the only other similar proteins ("Expect" score $<7\times10^{-7}$) were three paralogous proteins from each of the two sequenced *H. pylori* genomes, JHP0274 (accession C71953) and HP0289 (accession A64556), JHP0556 (accession D71917) and HP0609/0610 (accession A64596 and B64596), and JHP0856 (accession H71879) and HP0922 (accession B64635). HP0609 and HP0610 were identified as two adjacent open reading frames in the genome sequence of *H. pylori* strain 26695 (Tomb *et al*., 1997) and correspond to the single, larger open reading frame JHP0556 identified in strain J99 (Alm *et al*., 1999). In addition to these proteins from *H. pylori*, two proteins from *H. bilis*, designated p158 (accession AA039266) and p167 (accession AAQ14336) also exhibit similarity to VacA. A non-redundant set of proteins was compiled, and was then used as input into the Block Maker program (Henikoff *et al*., 1995), which finds "blocks" (short multiple aligned ungapped segments corresponding to the most highly conserved regions of proteins) within a group of related proteins. Related blocks are identified by shading, and the relative positions of the identified blocks in each related protein are depicted. The percent amino acid identity between VacA and each related protein, within the indicated regions corresponding to the carboxy-terminal autotransporter β-core domain, are listed. Less similar proteins were excluded from the Block Maker analysis.

natants from individual *H. pylori* strains. Another contributing factor is the high level of sequence diversity among different *vacA* alleles, resulting in VacA proteins with different amino acid sequences (Atherton *et al*., 1995). Analyses of VacA sequences from numerous *H. pylori* isolates have led to the recognition of several families of *vacA* alleles (Atherton *et al*., 1995; Strobel *et al*., 1998; van Doorn *et al*., 1998). Two families (s1 and s2) are recognized based on sequence variability at the 5′ end of the *vacA* gene, including the portion that encodes the VacA amino-terminal signal sequence (Figure 24.1B), and two additional families (m1 and m2) are recognized based on variability of *vacA* "midregions" (Figure 24.1C). Various s1, m1, and m2 subfamilies of *vacA* alleles have also been described. Among *H. pylori* isolates, s1/m1, s1/m2, and s2/m2 *vacA* alleles are relatively common and exhibit a worldwide distribution, whereas s2/m1 alleles are only rarely observed (Letley *et al*., 1999; Van Doorn *et al*., 1999). Surprisingly, the main families of *vacA* sequences (s1, s2, m1, and m2) have remained relatively intact despite evidence that recombination among *vacA* alleles from different strains has occurred commonly (Suerbaum *et al*., 1998; Atherton *et al*., 1999; Ji *et al*., 2002; Aviles-Jimenez *et al*., 2004). This suggests that various *in vivo* selective forces favor preservation of these structures.

VacA proteins that induce vacuolation of mammalian cells *in vitro* are expressed from s1-type alleles, whereas s2-type VacA proteins lack detectable vacuolating activity. The differences between s1- and s2-type VacA proteins are found both in the signal sequences (removed from the mature toxin) and in the amino-termini of the mature toxins. In comparison to s1-type toxins, s2-type proteins contain an additional 12 amino acids at the amino terminus (Atherton *et al*., 1995) (Figure 24.1B). Multiple studies have analyzed chimeric proteins in which an active s1-type VacA toxin was converted to a s2-type protein or vice versa (Letley and Atherton, 2000; McClain *et al*., 2001b; Letley *et al*., 2003). The results from these studies indicate that the 12 amino-acid extension found in s2-type VacA proteins confers a vacuolation-negative phenotype.

Mid-region diversity in *vacA* alleles has been linked to differences among VacA proteins in cell tropism (Pagliaccia *et al*., 1998; Ji *et al*., 2000; Wang *et al*., 2001). m1-type VacA toxins produce vacuolating cytotoxic effects in a wide variety of different cultured cell lines, including HeLa cells, which are commonly used to assess vacuolating cytotoxic activity. In contrast, m2-type VacA toxins appear to exhibit vacuolating activity toward a narrower range of cells (if they exhibit any activity) and are relatively inactive against HeLa cells (Pagliaccia *et al*., 1998). m2-type VacA toxins bind poorly to HeLa cells (Pagliaccia *et al*., 1998; Wang *et al*., 2001), which suggests that distinct cellular receptors for m1- and m2-type VacA toxins may account for the observed differences in cell tropism (Wang *et al*., 2001). Analysis of a series of m1/m2 chimeras has led to the identification of a stretch of 148 amino acids within the VacA m region that may determine cell specificity (Ji *et al*., 2000).

Based on the high level of genetic diversity among *vacA* alleles, it is not surprising that there is considerable antigenic variability among VacA toxins as well. Monoclonal antibodies produced against s1-m1 VacA failed to recognize s2-m2 VacA proteins (Vinion-Dubiel *et al*., 2001). This result is not completely unexpected because mature s1-m1 and s2-m2 VacA proteins are only about 75% identical in amino acid sequences overall and exhibit even greater amino acid diversity in the VacA m-region. More notable was the inability of various monoclonal antibodies to consistently recognize s1-m1 toxins from multiple different strains (Vinion-Dubiel *et al*., 2001). s1-m1 toxins produced by different *H. pylori* strains are typically about 90% identical in amino acid sequences. These data suggest that amino acid diversity among VacA proteins from different *H. pylori* strains may occur disproportionately in surface-exposed epitopes.

## ROLE OF VacA *IN VIVO*

### Mice and other model animals

Several studies have examined the role of VacA *in vivo* by using animal models for *H. pylori* infection. In one study, the *vacA* allele type of wild-type *H. pylori* isolates did not influence the ability of *H. pylori* to colonize mice, i.e., a variety of *H. pylori* strains were found to colonize independently of the *vacA* allele carried by each strain (Ayraud *et al*., 2002). This is consistent with the capacity of *H. pylori* strains containing various types of *vacA* alleles to colonize humans. However, the presence of VacA does appear to influence *H. pylori* colonization. When mice were co-infected with a mixture of *vacA*-positive and isogenic *vacA*-negative strains, the *vacA* knockout mutant did not colonize mice as efficiently as the wild-type parental strain (Salama *et al*., 2001). It is worth noting that the *H. pylori* strain, SS1, used in this study (Salama *et al*., 2001) has been reported by other investigators to contain an s2/m2 *vacA* allele (van Doorn *et al*., 1999a), which encodes a VacA protein with little if any cytotoxic activity *in vitro*. This suggests that critical functions of VacA remain to be determined. In a gerbil model, the colonization efficiency of a *vacA* mutant strain was slightly less than that of the parental

strain (Wirth *et al*., 1998), and no effect of VacA on colonization efficiency was detected in a gnotobiotic piglet model (Eaton *et al*., 1997).

Oral administration of purified VacA to mice causes gastric ulceration and inflammation (Telford *et al*., 1994; Fujikawa *et al*., 2003), accompanied by infiltration of both mast cells and monocytes (Supajatura *et al*., 2002). Based on the capacity of VacA to induce ulceration in mice when administered intragastrically, it has been suggested that VacA contributes to the pathogenesis of gastric ulceration in humans. However, the intragastric concentration of VacA in these experiments is likely to be substantially higher than that which is encountered in the context of human *H. pylori* infection.

### *H. pylori* infection of humans

In an effort to determine whether or not VacA plays an important role in *H. pylori* pathogenesis in humans, many studies have examined the relationship between *vacA* allele types and the development of gastroduodenal disease. Among *H. pylori*–infected individuals, infection with *H. pylori* strains containing an m1-type *vacA* allele is associated with an increased risk for gastric carcinoma compared to infection by strains containing an m2-type allele (Figueiredo *et al*., 2002). Additionally, strains containing an m1-type *vacA* allele are associated with more pronounced epithelial damage, neutrophilic and lymphocytic infiltration, atrophic gastritis, and intestinal metaplasia (Nogueira *et al*., 2001). Thus, in comparison to strains containing m2-type *vacA* alleles, strains containing m1-type *vacA* alleles appear to be associated with more severe disease outcome.

Studies have also found that infection with *H. pylori* strains containing *vacA* s1-type alleles seems to correlate with more severe clinical disease. Numerous studies have concluded that peptic ulceration occurs more commonly among patients infected with *H. pylori* strains containing an s1-type *vacA* allele than among patients infected with strains containing an s2-type *vacA* allele (Atherton *et al*., 1995; Han *et al*., 1998; Rudi *et al*., 1998; Strobel *et al*., 1998; Gerhard *et al*., 1999; Kidd *et al*., 1999; Van Doorn *et al*., 1999; De Gusmao *et al*., 2000). Similarly, s1-type VacA alleles are associated with an increased risk for gastric carcinoma (Figueiredo *et al*., 2002). These associations are less apparent in many Asian countries than in Europe and the Americas (Pan *et al*., 1998), perhaps because in many Asian countries, nearly all *H. pylori* isolates contain s1-type *vacA* alleles (Van Doorn *et al*., 1999). The correlation between vacA s-types and development of peptic ulcers in humans is consistent with studies linking s1-type VacA to gastric epithelial damage in animal models and with studies indicating that s2-type toxins lack activity in cell culture assays (Letley and Atherton, 2000; McClain *et al*., 2001b; Letley *et al*., 2003). Strains that contain s1-type *vacA* alleles more frequently contain the *cag* pathogenicity island and more frequently express the BabA adhesin than do strains that contain s2-type *vacA* alleles (Rudi *et al*., 1998; Gerhard *et al*., 1999), which suggests that multiple bacterial factors contribute to ulcerogenesis.

## *IN VITRO* ACTIVITIES

### Vacuole formation

One of the most striking activities of VacA is its capacity to cause formation of numerous, large cytoplasmic vacuoles in intoxicated cells (Leunk *et al*., 1988; de Bernard *et al*., 1998b) (Figure 24.3). VacA induces formation of vacuoles in many different transformed cell lines and also in primary cultures of human gastric epithelial cells (Smoot *et al*., 1996; de Bernard *et al*., 1998b). Vacuoles also have been observed in gastric epithelial cells in gastric biopsies from patients infected with *H. pylori* (Tricottet *et al*., 1986; Fiocca *et al*., 1992), but it is not clear whether these vacuoles are caused by VacA. VacA-induced vacuole formation *in vitro* is inhibited by monensin (an ionophore that dissipates intracellular proton gradients) (Cover *et al*., 1992c) and inhibitors of the vacuolar ATPase (v-ATPase), including bafilomycin A1 and an antibody specific to the v-ATPase (Cover *et al*., 1993b; Papini *et al*., 1993; Papini *et al*., 1996), indicating that the v-ATPase is required for VacA-induced vacuole formation. The lumen of VacA-induced vacuoles is acidic, as indicated by the ability of the vacuoles to sequester neutral red and acridine orange (Cover *et al*., 1991, Catrenich, 1992). The accumulation of neutral red is the basis for a widely-used

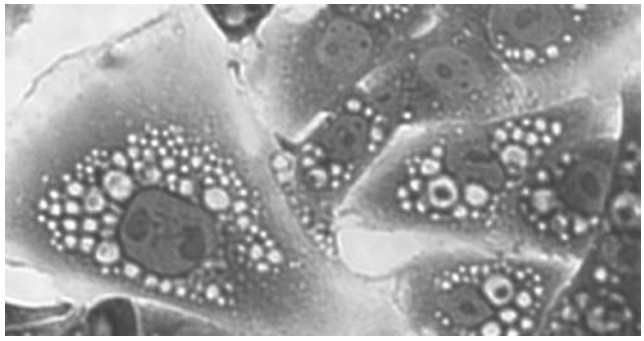


**FIGURE 24.3** VacA-induced vacuolation of HeLa cells. HeLa cell monolayers were treated with acid-activated VacA toxin purified from *H. pylori* culture supernatant in the presence of 5 mM $NH_4Cl$ and the cells were stained with crystal violet.

quantitative assay of the toxin's vacuolating activity (Cover *et al*., 1991). The formation of vacuoles in response to purified VacA is markedly stimulated by weak bases, such as ammonium salts, which enter acidic intracellular compartments in an uncharged state, become protonated, and may contribute to vacuole enlargement by inducing osmotic swelling (Cover *et al*., 1992c). *In vivo*, $NH_4^+$ ions may be supplied by the action of *H. pylori* urease, an essential enzyme required for the bacterium to survive in the acidic environment of the stomach, as well as by other pathways (Tombola *et al*., 2001). VacA-induced vacuoles possess characteristics typically associated with late endosomes and lysosomes, including the presence of rab7 (Papini *et al*., 1994), Lgp110 (Molinari *et al*., 1997), and Lamp1 (Li *et al*., 2004b) in the vacuole membrane, suggesting that VacA-induced vacuoles arise from these structures. Markers for other structures, including Golgi and early endosomes, have not been reported as components of VacA-induced vacuoles.

It is unlikely that increased osmotic pressure inside endocytic compartments can fully explain the swelling of VacA-induced vacuoles, as the increasing pressure would likely result in rupture of these compartments unless additional membrane were added to the growing vacuoles. It is likely that membrane fusion between nascent vacuoles and preexisting membrane-bound compartments contributes to vacuole biogenesis. Consistent with this model, vacuole formation was found to require the small GTP-binding protein Rab7 (Papini *et al*., 1997), which regulates fusion events between vesicles in the late endocytic pathway (Mohrmann and van der Sluijs, 1999). In addition, a recent study reported that syntaxin 7 is required for VacA-induced vacuole formation (Suzuki *et al*., 2003). Syntaxin 7 localizes to both late endosomes and lysosomes, and plays a crucial role in the fusion between late endosomes and lysosomes (Mullock *et al*., 2000; Nakamura *et al*., 2000). A requirement for syntaxin 7 and Rab7 is consistent with a model in which VacA-induced vacuole formation involves the docking and fusion of membrane vesicles via SNARE proteins.

In contrast, another study reported that microinjection of anti-syntaxin 7 antibodies into cells did not inhibit vacuole formation (de Bernard *et al*., 2002). This study also reported that cells transfected with a plasmid encoding dominant-negative α-SNAP did not vacuolate in response to exogenously added VacA, whereas, in contrast, these cells became vacuolated when cotransfected with a VacA-expressing plasmid. α-SNAP is an accessory protein that is essential for the recycling of SNARE protein complexes, allowing SNAREs to reform complexes that will drive subsequent rounds of membrane fusion (Whiteheart *et al*., 2001; Bonifacino and Glick, 2004). These results suggest that endocytosis of VacA requires α-SNAP, but that vacuole formation induced by intracellular VacA toxin does not require vesicle fusion via SNARE proteins. An alternative source of additional membrane for the nascent vacuoles may be found within late endosomes. Based on electron microscopy studies showing the lack of internal membrane structures within vacuoles (Catrenich and Chestnut, 1992; Cover *et al*., 1992b; Ricci *et al*., 1997; de Bernard *et al*., 2002), and on the apparent lack of lyso-bisphosphatidic acid (LBPA, a component of the internal membrane structures within endosomal and lysosomal membranes) in the lumen of VacA-induced vacuoles (de Bernard *et al*., 2002), it was proposed that the vacuoles may form by the addition of membrane from the extensive network of intracompartmental membranes within late endosomes (de Bernard *et al*., 2002).

If the process of vacuole formation involves fusion among preexisting membrane-bound compartments, how might VacA contribute to membrane fusion? VacA may function as a fusogenic protein in a manner analogous to a variety of viral fusion proteins (Nieva and Agirre, 2003; Li *et al*., 2004b). In this model, VacA is expected to become localized in the membrane of late endocytic compartments and function to promote docking or fusion of these compartments. In support of this hypothesis, VacA is internalized by cells and localizes in association with vacuole membranes (Sommi *et al*., 1998; Li *et al*., 2004b), and recombinant VacA fragments were shown to promote fusion of small unilamellar vesicles *in vitro* (Moll *et al*., 1995). Alternatively, VacA may modulate the activity of cellular proteins involved in intracellular pathways normally used for membrane fusion. Addition of purified VacA to cells in the absence of supplemental weak bases reveals a redistribution of several markers for late endocytic compartments to perinuclear clusters without the development of large cytoplasmic vacuoles (Li *et al*., 2004b). The membrane markers that undergo clustering and redistribution in response to VacA are the same as those that localize to the vacuole membrane in vacuolated cells, and experiments indicate that there is a structural relationship between VacA-induced perinuclear clusters and VacA-induced vacuoles (Li *et al*., 2004b). This VacA-induced clustering of late endocytic compartments resembles the clustering of late endocytic compartments observed following overexpression of constitutively active Rab7 (Bucci *et al*., 2000), RILP (a putative Rab7 effector) (Cantalupo *et al*., 2001), or Vam6p (the human homolog of a yeast protein that acts in conjunction with the yeast rab7 homolog in vesicle fusion in mammalian cells) (Nakamura *et al*., 1997; Caplan *et al*., 2001). Thus, VacA may act by modulating

the activity of Rab7 or related effector molecules, resulting in increased membrane fusion activity.

In addition to v-ATPase and Rab7, several other cellular constituents are required for the process of VacA-induced vacuole formation. These include dynamin and Rac1 (Hotchin *et al*., 2000; Suzuki *et al*., 2001). Disruption of microtubules by treatment with colchicine or nocodazole (Papini *et al*., 1994) and disruption of the actin cytoskeleton by treatment with cytochalasin D (Ricci *et al*., 2000) each result in at least partial inhibition of VacA-induced vacuolation (Li *et al*., 2004b). Overexpression of PIKfyve kinase (which produces phosphatidylinositol-3, 5-bisphosphate) inhibits VacA activity, which suggests that phosphatidylinositol derivatives may play an important role in VacA-induced cellular vacuolation (Ikonomov *et al*., 2002). In addition, cholesterol-rich lipid rafts, glycosylphosphatidylinositol (GPI)-anchored proteins, and the receptor protein tyrosine phosphatases (RPTP) RPTPα and RPTPβ have also been implicated in the process by which VacA induces vacuolation, and each is discussed in greater detail below (Yahiro *et al*., 1999; Ricci *et al*., 2000; Patel *et al*., 2002; Schraw *et al*., 2002; Kuo and Wang, 2003; Yahiro *et al*., 2003; Gauthier *et al*., 2004). The precise roles of all these cellular constituents in the process of VacA-induced vacuolation have not been completely elucidated. It seems likely that several of these factors may be required for binding, internalization, and intracellular trafficking of VacA, and other factors may be required for the process of vacuole biogenesis.

## Anion-conductive channels

VacA can form low-conductance, voltage-dependent, anion-selective channels in planar lipid bilayers (Czajkowsky *et al*., 1999; Iwamoto *et al*., 1999; Szabo *et al*., 1999; Tombola *et al*., 1999a). Anions such as $Cl^-$, $HCO_3^-$, and small organic molecules are conducted by VacA channels (Iwamoto *et al*., 1999; Tombola *et al*., 1999a). Evidence obtained from whole-cell, patch-clamp techniques indicates that VacA is able to form anion-selective channels in the plasma membrane of cells, leading to a partial depolarization of the membrane potential (Szabo *et al*., 1999). There is a correlation between the ability of VacA mutant proteins to form channels in lipid bilayers and the extent to which these proteins induce membrane depolarization of intact cells (McClain *et al*., 2003). These results support the conclusion that the plasma membrane channels formed in response to VacA are indeed directly due to the toxin, as opposed to the activation of a cellular channel in response to the toxin. The channels formed by VacA in the plasma membrane can lead to the diffusion of small ions, including $Cl^-$, $HCO_3^-$, and urea, across the cell membrane (Szabo *et al*., 1999; Debellis *et al*., 2001; Tombola *et al*., 2001).

VacA mutants that exhibit defects in the capacity to form membrane channels in planar lipid bilayers invariably lack vacuolating cytotoxic activity (Tombola *et al*., 1999a; Vinion-Dubiel *et al*., 1999; McClain *et al*., 2001b; McClain *et al*., 2003). Furthermore, a variety of chemicals that inhibit chloride channel activity also inhibit the ability of VacA to form channels and to induce vacuolation (Iwamoto *et al*., 1999; Szabo *et al*., 1999; Tombola *et al*., 1999b).The relative strength of the VacA channel inhibitors correlates with the ability of the chemicals to inhibit vacuolation (Tombola *et al*., 1999b). In addition, the histidine-modifying reagent diethylpyrocarbonate (DEPC), which inhibits vacuolating activity, was also found to inhibit VacA channels (Tombola *et al*., 1999a). Taken together, these data support the hypothesis that VacA channel activity is required for vacuole formation. However, channel formation alone does not appear to be sufficient to induce vacuole formation, as certain dominant-negative mutants interfere with the vacuolating activity of wild-type VacA, but do not appear to interfere with channel activity (McClain *et al*., 2001b).

Tombola *et al*. (Tombola *et al*., 1999a) proposed a model to account for the relationship between VacA channel activity and vacuolation (Figure 24.4). This model proposes that VacA is internalized by cells and localizes to the membrane of endocytic compartments, and that VacA channels are formed in this site. The formation of VacA channels in the membrane of

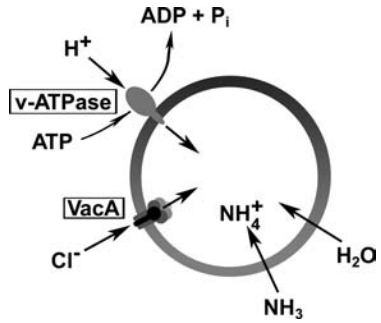



**FIGURE 24.4** VacA channel activity is required for vacuolation. In this model (Tombola *et al*., 1999a), the VacA toxin inserts into the membrane of endosomes to form anion-selective membrane channels. The influx of $Cl^-$ through the VacA channel presumably activates the v-ATPase (Al-Awqati, 1986), leading to an increase in the lumenal concentration of $H^+$. Weak bases such as $NH_3$ diffuse across the vacuole membrane and become protonated and sequestered, leading to an osmotically driven influx of $H_2O$, and resulting in an increased osmotic pressure within the vacuole. The increased pressure is presumed to help drive enlargement of the vacuolar compartment.

v-ATPase–containing vesicles is expected to lead to an influx of $Cl^-$ into the vesicle lumen. The increased intralumenal $Cl^-$ concentration is expected to stimulate the v-ATPase (Al-Awqati, 1986), resulting in an increase in lumenal $H^+$. This acidification of the vesicle leads to the influx of weak bases (such as $NH_3$), which become protonated and accumulate within the vesicle, resulting in the influx of water to counteract the increased tonicity. In support of this model, vacuolation is accompanied by and requires uptake of $Cl^-$ from the medium, and vacuolation can be inhibited by hyperosmotic medium (Morbiato *et al*., 2001).

Endogenous chloride channels are found in a variety of intracellular organelles, including endosomes and lysosomes, where the channels are believed to function as an electrical shunt for the v-ATPase (al-Awqati, 1995; Szewczyk, 1998). Proton pumping by the v-ATPase involves the transfer of both acid equivalents and electrical charge across the vesicle membrane, and $H^+$ entry into the lumen must be accompanied by anion entry (or cation exit) in order to dissipate the electrical potential across the membrane. Acidification of endosomes by the v-ATPase is accompanied by significant increases in both intralumenal $Cl^-$ concentrations and in endosome volume (Sonawane *et al*., 2002). Overexpression of the intracellular chloride channel ClC-3 in human hepatoma (Huh-7) cells and in Chinese hamster ovary (CHO-K1) cells induces the formation of large cytoplasmic vacuoles with characteristics of late endosomes or lysosomes, including the presence of Lamp-1 on the vacuole membranes (Li *et al*., 2002). Furthermore, the formation of the ClC-3–induced vacuoles requires the channel activity of the ClC-3 protein, and is inhibited by bafilomycin A1, suggesting a role for the v-ATPase (Li *et al*., 2002). Thus, overexpression of ClC-3 results in cytoplasmic vacuolation that is remarkably similar to the vacuolation induced by the VacA toxin. This similarity is consistent with the hypothesis that membrane channels play an important role in VacA-induced vacuole formation.

## Effects on mitochondria and apoptosis

Addition of VacA to cells results in perturbations to mitochondria, as evidenced by a decrease in cellular ATP levels (Kimura *et al*., 1999), a decrease in the mitochondrial membrane potential (Kimura *et al*., 1999; Willhite and Blanke, 2004), and the release of cytochrome c (Galmiche *et al*., 2000; Willhite *et al*., 2003). The VacA-induced effects on mitochondria appear to depend on VacA channel activity, as both the VacA-induced reduction in mitochondrial membrane potential and the release of cytochrome c are inhibited by chemicals that block VacA channels (NPPB and DIDS) (Willhite *et al*., 2003) and by a channel-inhibiting, dominant-negative mutant VacA protein (Vinion-Dubiel *et al*., 1999; Willhite *et al*., 2003). Furthermore, VacA mutants that lack channel activity do not cause release of cytochrome c from mitochondria (Willhite *et al*., 2003). The effects of VacA on mitochondria seem to be independent of vacuolating activity, as mitochondrial damage is not inhibited by bafilomycin A1 (Willhite *et al*., 2003), and the VacA-induced mitochondrial effects occur at higher toxin concentrations and at later time points than are required to induce vacuolation (Willhite *et al*., 2003; Willhite and Blanke, 2004).

VacA expressed within transfected cells or added exogenously to cultured cells has been observed to associate with mitochondria (Galmiche *et al*., 2000; Willhite and Blanke, 2004). In experiments in which VacA was incubated with isolated mitochondria, the toxin was found to remain free within the matrix or associated with the inner mitochondrial membrane (Galmiche *et al*., 2000). Just as the formation of channels by VacA in the endocytic membrane could lead to vacuole formation, the formation of channels by VacA in the inner mitochondrial membrane might contribute directly to a decrease in the mitochondrial membrane potential and the release of cytochrome c. Thus, VacA might act in a manner analogous to the mitochondrial permeability transition pore (PT) (Chang and Yang, 2000; Willhite and Blanke, 2004). Opening of the PT pore causes swelling of the mitochondrial matrix (due to the high osmolarity of the matrix), leading to rupture of the mitochondrial outer membrane and the release of cytochrome c. The disruption of the inner mitochondrial membrane potential has also been associated with opening of the PT pore. It is not clear how endocytosed VacA finds its way to the mitochondria. It has been suggested that some VacA protein might undergo trafficking from the endocytic pathway retrograde through the Golgi to the cytoplasm (Argent *et al*., 2004), and could thus gain access to the mitochondria. However, mechanisms by which VacA might cross the outer mitochondrial membrane remain unclear.

Exogenously added VacA, as well as various VacA fragments that are expressed in transiently transfected HeLa cells, induce cleavage of poly(ADP-ribose) polymerase (PARP), a substrate of activated caspase-3 (Tewari *et al*., 1995; Galmiche *et al*., 2000; Cho *et al*., 2003). Caspase 3 is one of the effector caspases, which cleave various cytoplasmic and nuclear proteins (including PARP), thereby triggering apoptosis (Chang and Yang, 2000). One pathway leading to the activation of caspase-3 is the release of cytochrome c from mitochondria. Cytosolic cytochrome c forms a complex with procaspase-9 and apoptotic protease activating

factor 1 (APAF-1), leading to activation of caspase-9, which in turn activates the effector caspases, including caspase-3 (Chang and Yang, 2000).

Consistent with the findings that VacA induces perturbations in mitochondria and cleavage of PARP, analyses of wild-type and *vacA* mutant *H. pylori* strains have indicated that VacA contributes to the ability of *H. pylori* to induce apoptosis of cultured cell lines (Peek *et al*., 1999; Kuck *et al*., 2001; Menaker *et al*., 2004). Further experiments have demonstrated that purified VacA can induce apoptosis in the absence of other *H. pylori* factors (Kuck *et al*., 2001; Cover *et al*., 2003). A mutant VacA protein that lacks channel-forming activity failed to induce apoptosis (Cover *et al*., 2003), which suggests that VacA-induced apoptosis is a process that requires the formation of VacA channels.

## Immune-modulating activities

In addition to stimulating the formation of cytoplasmic vacuoles and contributing to apoptosis, VacA interferes with the activation and proliferation of T lymphocytes. VacA is able to inhibit T lymphocyte activation and proliferation by a variety of different mechanisms. First, VacA blocks activation of nuclear factor of activated T cells (NFAT), a key transcription factor required for optimal T lymphocyte activation (Boncristiano *et al*., 2003; Gebert *et al*., 2003). This effect has been attributed to abrogation of calcium influx, thereby blocking the activity of the $Ca^{2+}$-calmodulin-dependent phosphatase calcineurin (Gebert *et al*., 2003). Second, VacA inhibits IL-2-dependent cell-cycle progression and proliferation via a mechanism that is independent of VacA effects on NFAT activation (Sundrud *et al*., 2004). Both of these mechanisms appear to depend on VacA channel-forming activity, as mutant VacA toxins that lack channel activity do not inhibit T cell proliferation (Boncristiano *et al*., 2003; Sundrud *et al*., 2004), and the inhibition of cell cycle progression by wild-type VacA is blocked by a channel-inhibiting, dominant-negative mutant VacA (Vinion-Dubiel *et al*., 1999; Sundrud *et al*., 2004). Finally, VacA can interfere with T cell activation through a channel-independent mechanism that involves activation of intracellular signaling through the mitogen-activated protein kinases MKK3/6 and p38 and the Rac-specific nucleotide exchange factor, Vav (Boncristiano *et al*., 2003).

VacA has been demonstrated to produce several additional effects on immune cells. *H. pylori* strains expressing VacA, in contrast to isogenic *vacA* null mutants, inhibit phagosome-lysosome fusion in macrophages (Zheng and Jones, 2003). Phagosomes containing VacA-expressing *H. pylori* were found to retain EEA1, a marker of early endosomes, and had low levels of LAMP1 (a lysosomal marker), whereas the phagosomes containing a *vacA* mutant strain had lower levels of EEA1 and higher levels of LAMP1 (Zheng and Jones, 2003). The phagosomes containing the VacA-expressing *H. pylori*, but not phagosomes containing the mutant *H. pylori*, also retained the tryptophan aspartate-containing coat protein (TACO) (Zheng and Jones, 2003), retention of which has been linked to defective trafficking of phagosomes containing *Mycobacterium bovis* (Ferrari *et al*., 1999). VacA was also shown to inhibit antigen presentation using a model system consisting of tetanus-toxoid-specific human $CD4^+$ T cells and autologous B cells (Molinari *et al*., 1998b). Specifically, antigen presentation via newly synthesized class II MHC molecules was inhibited by VacA, but VacA did not inhibit presentation relying upon recycled MHC class II molecules. Peptide-class II complexes are assembled in endocytic, lysosome-like compartments, where newly synthesized class II molecules are targeted from the trans-Golgi network (TGN) (Hiltbold and Roche, 2002).

The ability of VacA to inhibit both phagosome-lysosome fusion and antigen presentation suggests that the toxin induces a generalized defect in endocytic trafficking in cells exposed to the toxin. In support of this hypothesis, VacA-treated HeLa cells exhibit a variety of defects related to altered endocytic trafficking, including inhibited maturation of procathepsin D and inhibition of epidermal growth factor degradation (Satin *et al*., 1997). The observed defects in endocytic trafficking are associated with a partial neutralization of the pH inside endosomes and lysosomes (Satin *et al*., 1997).

VacA also produces pro-inflammatory effects on several cell types, including mast cells. *In vivo*, significant numbers of mast cells are observed in the gastric mucosa of patients infected with *H. pylori* (Nakajima *et al*., 1997; Nakajima *et al*., 2004), and similar mast cell infiltration is observed in mice experimentally colonized with *H. pylori* (van Doorn *et al*., 1999b). Within three minutes after the addition of VacA to a cultured mast cell line *in vitro*, the cytoplasmic $Ca^{2+}$ concentration was observed to undergo rapid oscillations that lasted in excess of two hours, and similar results were reported using primary, bone-marrow derived mouse mast cells (de Bernard *et al*., 2004). VacA induces mast cell degranulation (de Bernard *et al*., 2004), and stimulates mast cells to secrete pro-inflammatory cytokines, including tumor necrosis factor $\alpha$ in a $Ca^{2+}$-dependent mechanism (Supajatura *et al*., 2002; de Bernard *et al*., 2004). Furthermore, VacA has been reported to stimulate the production of the pro-inflammatory enzyme cyclooxygenase 2 (Cox-2) in the MKN-28 gastric cancer

cell line (Caputo *et al*., 2003) and in human neutrophils and macrophages (Boncristiano *et al*., 2003).

### Other activities

Human gastric epithelial cell lines treated with VacA exhibit decreased thymidine incorporation (Ricci *et al*., 1996; Pai *et al*., 2000; Tabel *et al*., 2003) and an increased percentage of cells in the $G_0/G_1$ phase of the cell cycle compared with untreated control cells (Kimura *et al*., 1999). VacA has also been shown to inhibit the migration of cells into wounds introduced in cultured monolayers (Tabel *et al*., 2003). The reduced wound healing observed in monolayers treated with the toxin correlates with the ability of VacA to disrupt the actin cytoskeleton (Pai *et al*., 1999; Ashorn *et al*., 2000; Tabel *et al*., 2003). Thus, the ability of VacA to inhibit cell migration and proliferation, both of which are required for maintenance of the gastric epithelium and for ulcer healing, may directly impact ulcerogenesis.

When added to polarized epithelial monolayers, VacA increases the trans-epithelial resistance (TER) and the paracellular permeability to small molecules, including $Fe^{3+}$ and $Ni^{2+}$, that are essential for *H. pylori* survival *in vivo* (Papini *et al*., 1998). Significantly, these effects of VacA were also observed when *H. pylori* strains expressing m2-type VacA proteins, which do not induce vacuoles in the cell line tested, were added to polarized monolayers, but were not observed when *vacA* null mutant strains were added (Pelicic *et al*., 1999). The ability of VacA to increase TER does not require the addition of weak bases and is not blocked by inhibitors of the v-ATPase, but is inhibited by the chloride channel inhibitor NPPB. This suggests that VacA channels might be mechanistically involved in this phenomenon via a pathway distinct from that which leads to vacuole formation.

Purified VacA induces phosphorylation of the MAP kinases Erk1/2 and p38 in a gastric epithelial cell line (Nakayama *et al*., 2004), and phosphorylation of p38 in the Jurkat T-cell line and in human macrophages and neutrophils (Boncristiano *et al*., 2003). VacA-induced phosphorylation of p38 was shown to lead to activation of p38 kinase activity, leading to the activation of downstream effectors in several of these cell types (Boncristiano *et al*., 2003; Nakayama *et al*., 2004). Among cells treated with a specific inhibitor of p38 kinase activity, VacA continued to induce vacuolation and led to a decrease in mitochondrial membrane potential and the release of cytochrome c (Nakayama *et al*., 2004). Thus, these phenomena occur via mechanisms that are distinct from the p38 signaling pathway. In addition to effects of VacA on MAP kinase signaling pathways, VacA has been reported to alter several additional signaling pathways in various cell types (Pai *et al*., 2000; Asahi *et al*., 2003; Boncristiano *et al*., 2003; Caputo *et al*., 2003; Fujikawa *et al*., 2003; Suzuki *et al*., 2003; Yuan *et al*., 2004).

## VacA STRUCTURE AND FUNCTION

### Formation of oligomeric structures

Early studies of VacA indicated that the toxin could assemble into high molecular mass structures (Cover and Blaser, 1992). Imaging of purified VacA by deep-etch electron microscopy (EM) indicates that VacA monomers assemble into flower-shaped oligomeric structures comprised of 6 to 14 subunits (Lupetti *et al*., 1996; Cover *et al*., 1997; Lanzavecchia *et al*., 1998) (Figure 24.5). The predominant oligomer appears to be two hexamers stacked to form a dodecamer. These structures are about 30 nm in diameter, and consist of a central ring surrounded by peripheral "arms" or "petals." Imaging of VacA in a vitrified aqueous environment by EM with cryonegative staining (Adrian *et al*., 2002) has permitted visualization of side views of these structures, in which the petals are slightly separated from each other, and the closest contact between the two hexamers appears to occur between the central rings. This suggests that the central ring is the most hydrophobic portion of the complex, while

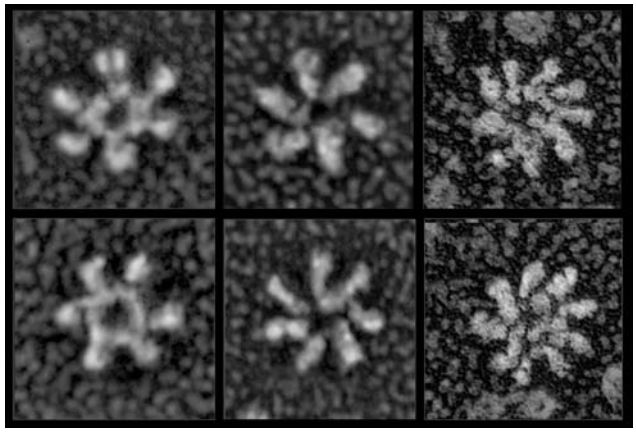


**FIGURE 24.5** Deep-etch electron micrographs of purified oligomeric VacA absorbed to mica flakes. Several studies have examined the organization of VacA oligomers by electron microscopy (Lupetti *et al*., 1996; Cover *et al*., 1997; Lanzavecchia *et al*., 1998; Adrian *et al*., 2002). Left column: Radially symmetric "flowers" exhibiting a prominent central ring. Middle column: Flatter forms lacking a prominent central ring. Right column: VacA oligomers absorbed to mica were treated with pH 3.5 glycine buffer to reveal dissociation into monomeric components.

the petals may be relatively hydrophilic (Adrian *et al*., 2002).

The structure of VacA oligomers associated with a lipid membrane appears slightly different from the structure of VacA oligomers observed in solution. Imaging of VacA bound to lipid membranes by both deep-etch EM and atomic-force microscopy (AFM) reveals that VacA assembles predominantly into single-layered hexameric rings with a central pore (Czajkowsky *et al*., 1999; Iwamoto *et al*., 1999; Adrian *et al*., 2002). Correspondingly, the kinetics of VacA channel formation in planar lipid bilayers suggests that VacA forms hexameric structures (Iwamoto *et al*., 1999).

In cells transfected with genes expressing CFP- and YFP-tagged VacA, the toxin was shown to assemble into oligomeric complexes, based on fluorescence resonance energy transfer (FRET) analysis (Willhite *et al*., 2002). Additional studies have shown a correlation between the formation of VacA oligomeric complexes and VacA activity. First, an analysis of mutant toxins indicated that toxins that fail to oligomerize also do not induce vacuolation (Vinion-Dubiel *et al*., 1999). Second, certain inactive mutant VacA toxins, when expressed together inside transfected cells, complement each other to induce vacuole formation (Ye and Blanke, 2002). Finally, certain inactive mutant toxins exhibit a dominant-negative phenotype when mixed with wild-type VacA (Vinion-Dubiel *et al*., 1999; McClain *et al*., 2001b). Evidence suggests that this dominant-negative activity is due to the formation of mixed-oligomeric complexes containing both wild-type and mutant VacA proteins (McClain *et al*., 2001b). It remains to be determined whether VacA binds to cells as an oligomeric "prepore" that subsequently inserts into the membrane, or whether the active toxin oligomer assembles from monomers that have already bound to cells or inserted into the membrane.

The oligomeric VacA toxin purified from *H. pylori* culture supernatant exhibits relatively little vacuolating cytotoxic activity, but its activity can be greatly enhanced if the purified protein is first acidified or alkalinized before it is added to cells (de Bernard *et al*., 1995; Yahiro *et al*., 1999). Acid- or alkaline-activation of the VacA toxin results in structural changes as evidenced by changes in circular dichroism, fluorescence spectra, and labeling with the hydrophobic probe 1-anilino-8-naphthalenesulfonate (ANS) (de Bernard *et al*., 1995; Molinari *et al*., 1998a). These structural changes are accompanied by a disruption of the VacA oligomeric structure into component monomers (Cover *et al*., 1997; Molinari *et al*., 1998a; Yahiro *et al*., 1999). The disrupted monomers are able to reassemble into oligomeric structures if the pH is returned to neutrality (Cover *et al*., 1997). Thus, it is believed that activated VacA monomers may reassemble into toxin oligomers when added to the neutral pH medium overlying cells in culture (though it is unclear whether such reassembly occurs in solution or on the cell surface). VacA in crude culture supernatants does not require activation to exhibit vacuolating cytotoxic activity, which suggests that the striking effect of these pH shifts may be limited to experiments involving purified oligomeric VacA. Nevertheless, the phenomenon of activation has contributed significantly to our understanding of VacA structure-function relationships. For example, compared with non-activated purified VacA, activated toxin exhibits increased binding to the cell surface, and only activated VacA is internalized by cells (McClain *et al*., 2000; Schraw *et al*., 2002).

## VacA domains

The 88-kDa VacA monomer can be proteolytically processed into an amino-terminal 33-kDa (p33) and a carboxy-terminal 55-kDa (p55) fragment (Nguyen *et al*., 2001). The reported masses of these fragments vary considerably in the literature, and these differences may be due to sequence variation among *vacA* alleles in different *H. pylori* strains. It has been suggested that the p33 and p55 fragments represent two domains of VacA. The cleavage of 88-kDa VacA into 33- and 55-kDa fragments can be detected in highly purified VacA preparations, but it is not known whether the cleavage results from an autoproteolytic activity of VacA or from a contaminating protease. Cleavage of 88-kDa VacA into these two fragments has no detectable impact on vacuolating activity, and the two fragments remain physically associated (Lupetti *et al*., 1996; Torres *et al*., 2004). Co-transfection of cells with genes encoding both p33 and p55 leads to vacuolation, whereas each fragment, individually, is inactive (Ye *et al*., 1999); this suggests that at least a portion of each fragment is required for toxin activity. Evidence indicates that independently expressed recombinant p33 and p55 fragments, when mixed together, can physically interact. The p33 and p55 fragments were shown to interact inside transfected cells by co-immunoprecipitation and by FRET analysis (Willhite *et al*., 2002; Ye and Blanke, 2002). Further studies of these two fragments by yeast-two hybrid analysis revealed interactions between p33 and p55, but interactions between p33 and p33 or between p55 and p55 were not detected in this system (Torres *et al*., 2004).

By transfecting the *vacA* gene into cultured cell lines, researchers have been able to define portions of the VacA toxin that are essential for vacuolating activity (de Bernard *et al*., 1997; de Bernard *et al*., 1998a; Ye *et al*.,

1999; Ye and Blanke, 2002). Such transfection studies have clearly indicated the importance of the amino-terminal portion of VacA. Deletion of as few as five amino acids from the amino terminus of VacA is sufficient to eliminate vacuolating activity (de Bernard *et al*., 1998a). The minimal fragment of VacA that is able to induce cytoplasmic vacuolation when expressed inside cells comprises the amino-terminal 422 amino acids of the toxin (Ye *et al*., 1999). This portion of VacA corresponds to the entire p33 fragment and the first 111 amino acids of the p55 fragment. Whereas intracellular expression of the p33 VacA fragment did not induce vacuolation, this fragment was active when co-expressed with a fragment corresponding to amino acids 312 to 478 (the amino-terminal 167 amino acids of p55) (Ye *et al*., 1999).

Amino acid sequences near the amino terminus of VacA play an important role in both vacuolation and membrane channel formation (de Bernard *et al*., 1998a; Ye *et al*., 1999; Letley and Atherton, 2000; Ye and Blanke, 2000; McClain *et al*., 2001b; Letley *et al*., 2003; McClain *et al*., 2003). The amino terminal end of s1-type VacA toxins is predicted to be hydrophobic, and appears to be the only contiguous stretch of hydrophobic amino acids long enough to span a membrane (Figure 24.6). The ability of this region to insert into a membrane has been shown using the TOXCAT system (designed to detect transmembrane helix association in a biological membrane) in *E. coli* (Russ and Engelman, 1999; McClain *et al*., 2001a). Based on this result and on the analysis of VacA toxins containing single amino acid substitutions within the hydrophobic region, it seems likely that this amino-terminal hydrophobic region of VacA either forms part of the VacA channel or plays an indirect role by stabilizing the structure of the channel (McClain *et al*., 2003). An important feature of the VacA amino-terminal hydrophobic region is the presence of GXXXG motifs, which are associated with packing interfaces among protein transmembrane domains (Russ and Engelman, 2000; McClain *et al*., 2003). The significance of the GXXXG motif has been most widely studied using glycophorin A as a model transmembrane protein (Lemmon *et al*., 1992; Lemmon *et al*., 1994; MacKenzie *et al*., 1997; Brosig and Langosch, 1998; Russ and Engelman, 2000). In addition, the importance of GXXXG motifs has been reported in a variety of other transmembrane proteins (Asundi and Carey, 1995; Williams *et al*., 1995; Hebert *et al*., 1996; Mendrola *et al*., 2002; Arselin *et al*., 2003; Lee *et al*., 2004; Li *et al*., 2004a; Polgar *et al*., 2004) and in soluble proteins as well (Kleiger *et al*., 2002). The VacA hydrophobic region contains three tandem copies of the GXXXG motif (Figure 24.6A). One copy in particular, comprised of glycines at positions 14 and 18, has been shown to be important in the packing interface of this putative VacA transmembrane domain as determined by the TOXCAT system, and is essential for both vacuolating activity and membrane channel formation (McClain *et al*., 2003). A recent theoretical model of the VacA amino terminus is consistent with the $G_{14}XXXG_{18}$ repeat assembling into the most closely packed region of the structure (Figure 24.6 B and C) (Kim *et al*., 2004).

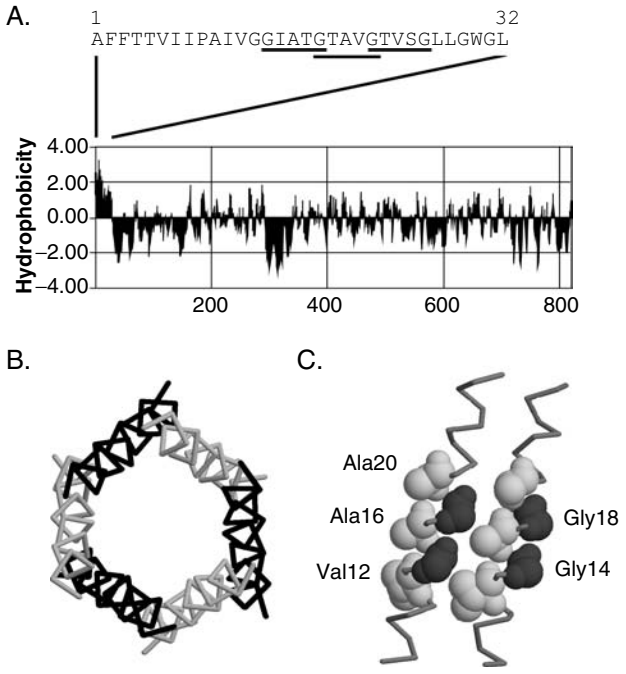



**FIGURE 24.6** The amino terminus of s1-type VacA**.** A. Based on a hydrophobicity plot of the amino acid sequence of VacA from *H. pylori* strain 60190, the amino terminal 32 amino acids of VacA are predicted to form a hydrophobic region. Three tandem copies of a "GXXXG" motif ($G_{14}XXXG_{18}$, $G_{18}XXXG_{22}$, and $G_{22}XXXG_{26}$) are found within this hydrophobic region. B and C. A theoretical model proposes that the amino terminal hydrophobic regions from six individual VacA monomers adopt an α-helical configuration and pack together to form a six-membered ring (Kim *et al*., 2004). This structure is shown from the top in panel B. Two adjacent helices from the proposed structure are shown in a side view (i.e., in a cross section of the membrane) (panel C) with space-filling representations of glycines 14 and 18 (dark shaded) and valine 12 and alanines 16 and 20 (light shaded) forming the most closely packed region.

Whereas much of the intracellularly active domain of VacA appears to reside within the p33 fragment, the p55 fragment appears to play a significant role in binding of the toxin to cells. Antibodies raised against the p55 fragment, but not antibodies against the p33 fragment, block binding of VacA to cells (Garner and Cover, 1996), and a p55 fragment expressed in *H. pylori* is able to bind to cells (Reyrat *et al*., 1999). Curiously, this p55 fragment was not internalized by cells (Reyrat *et al*., 1999), suggesting a need for the p33 domain for toxin internalization. VacA proteins of the m1 and m2

types (which differ in amino acid sequences mainly within the p55 domain) exhibit distinct cell tropisms, which are due (at least in part) to differences in binding to cells (Pagliaccia *et al*., 1998; Wang *et al*., 2001). One region of the p55 domain important for binding was mapped by analysis of recombinant GST-VacA fusion proteins (Wang and Wang, 2000). This study concluded that the last 100 amino acids of VacA are critical for the ability of VacA to bind to cells. This region is distinct from sequences reported to be responsible for the cell tropism of m1- and m2-type VacA proteins (Ji *et al*., 2000).

## INTERACTIONS WITH HOST CELLS

### Interactions at the cell surface

Several previous studies have provided evidence for saturability and specificity of VacA binding to cells (Massari *et al*., 1998; Wang and Wang, 2000; Wang *et al*., 2001). In contrast, studies with radiolabeled VacA have not provided convincing evidence of a saturable binding process, and the binding of radiolabeled VacA was only partially inhibited by excess unlabeled VacA (McClain *et al*., 2000; Ricci *et al*., 2000; Patel *et al*., 2002). At least five different putative receptors for VacA have been reported, including receptor protein tyrosine phosphatase β (RPTPβ) (Yahiro *et al*., 1999), RPTPα (Yahiro *et al*., 1997; Yahiro *et al*., 2003), the epidermal growth factor receptor (Seto *et al*., 1998), heparan sulfate (Utt *et al*., 2001), and various lipids (Moll *et al*., 1995; Molinari *et al*., 1998a; Czajkowsky *et al*., 1999; Geisse *et al*., 2004).Thus, at present, there is considerable confusion about which cell surface components are most relevant for VacA binding and cytotoxicity. It remains unclear whether VacA cytotoxicity is dependent on binding of VacA to one specific receptor, or whether cytotoxicity results from specific or nonspecific binding of VacA to multiple cell-surface components.

The most thoroughly characterized interaction between VacA and a putative receptor is the interaction between the toxin and RPTPβ (Yahiro *et al*., 1999; Padilla *et al*., 2000; Fujikawa *et al*., 2003). This interaction was initially characterized by observing that acid- or alkaline-activated VacA, but not the inactive oligomeric form of the purified toxin, could associate with RPTPβ expressed on the surface of AZ521 gastric epithelial cells (Yahiro *et al*., 1999). An important set of experiments was subsequently performed using HL60 cells (Padilla *et al*., 2000). This cell line is normally relatively resistant to VacA but becomes sensitive following differentiation into macrophage-like cells (de Bernard *et al*., 1998c). Both induction of VacA sensitivity and expression of RPTPβ mRNA were directly dependent on the concentration and time of exposure of cells to the differentiating agent phorbol 12-myristate 13-acetate (PMA) (Padilla *et al*., 2000). Treatment of HL-60 cells with RPTPβ antisense oligonucleotide before incubation with PMA led to a dose-dependent decrease in VacA sensitivity. In this same study, RPTPβ cDNA was stably expressed in BHK-21 cells, a cell line that is normally resistant to VacA (Padilla *et al*., 2000). BHK-21 cells expressing RPTPβ showed greater sensitivity to VacA than did control cells or cells transfected with vector only. Compelling evidence that RPTPβ plays a significant role in VacA intoxication was provided by studies using transgenic RPTPβ −/− mice (Fujikawa *et al*., 2003). In wild-type C57BL/6 mice, oral administration of either VacA or the endogenous RPTPβ ligand pleiotrophin induced disruption of the gastric epithelium, whereas neither VacA nor pleiotrophin disrupted the epithelium in RPTPβ −/− mice. Furthermore, primary gastric cells from wild-type mice detached from a reconstituted basement membrane following VacA treatment, whereas cells from the RPTPβ −/− mice did not detach (Fujikawa *et al*., 2003). However, VacA causes vacuolation of cells that do not express RPTPβ, including primary gastric cells from RPTPβ−/− mice and the human kidney cell line G401, indicating that RPTPβ is not the only receptor for VacA (Fujikawa *et al*., 2003; Yahiro *et al*., 2003).

A second VacA-interacting protein, p140, was detected in the gastric cell lines AZ-521 and AGS, as well as in the monkey kidney line COS-7 (Yahiro *et al*., 1997). Internal amino acid sequence analysis and time of flight-mass spectrometry analysis of p140 identified this protein as RPTPα (Yahiro *et al*., 2003). Treatment of G401 cells (which do not express detectable RPTPβ) with RPTPα-antisense oligonucleotide before exposure to VacA inhibited vacuolation (Yahiro *et al*., 2003). These results support the involvement of RPTPα in VacA-induced vacuolation in G401 cells.

Most RPTPs, including RPTPα and RPTPβ, contain two cytoplasmic protein tyrosine phosphatase (PTP) domains, a single transmembrane segment, and an extracellular domain (Beltran and Bixby, 2003). Though the cytoplasmic PTP domains are highly conserved, the extracellular domains of RPTPs are quite variable. Similarity between the extracellular domains of RPTPα and RPTPβ is limited to a short segment of each protein (less than 30% amino acid identity over a stretch of about 50 amino acids) (Yahiro *et al*., 2003), but a recent study implicates an unrelated region of RPTPβ as being critical for the interaction with VacA (Yahiro *et al*., 2004).

Multiple studies have documented that VacA associates with cholesterol-rich microdomains (rafts) on the surface of epithelial cells (Patel *et al*., 2002; Schraw *et al*., 2002; Kuo and Wang, 2003; Gauthier *et al*., 2004). VacA interactions with lipid rafts have also been detected *in vitro*, based on AFM imaging (Geisse *et al*., 2004). The association of purified VacA oligomers with rafts requires activation of the toxin by exposure to acidic pH (Schraw *et al*., 2002). Depletion of cholesterol from cells by treatment with methyl-β-cyclodextrin disrupts raft integrity and also inhibits VacA-induced vacuolation (Patel *et al*., 2002; Schraw *et al*., 2002; Kuo and Wang, 2003). Specifically, cholesterol depletion partially inhibits VacA binding to cells and blocks VacA internalization or intracellular trafficking (Patel *et al*., 2002; Schraw *et al*., 2002; Kuo and Wang, 2003). However, cholesterol depletion does not appear to inhibit the ability of VacA to form channels within the plasma membrane as determined by measuring membrane depolarization (Schraw *et al*., 2002). The inhibition of vacuolating activity by cholesterol depletion is also observed in cells pretreated with VacA, as well as in cells in which the *vacA* gene is transfected, suggesting that cholesterol also plays an important role after VacA has been internalized (Patel *et al*., 2002). Cholesterol-rich rafts can be isolated from late endosomes (Fivaz *et al*., 2002), where cholesterol modulates the rab7 activity cycle (Lebrand *et al*., 2002), possibly accounting for the role of intracellular cholesterol in VacA-induced vacuolation (Patel *et al*., 2002).

Cholesterol-rich membrane rafts are enriched in glycosylphosphatidylinositol (GPI)-anchored proteins, and treatment of cells with phosphatidylinositol-specific phospholipase C (PIPLC) removes GPI-anchored proteins from the cell surface (Sharom and Lehto, 2002). Cells treated with PIPLC have been found to be relatively resistant to VacA-induced vacuolation (Ricci *et al*., 2000; Kuo and Wang, 2003; Gauthier *et al*., 2004). However, the role of GPI-anchored proteins in VacA toxicity is unclear. Removal of GPI-anchored proteins by PIPLC treatment has no detectable effect on VacA binding to cells or on VacA localization to rafts (Kuo and Wang, 2003; Gauthier *et al*., 2004). Additionally, mutant CHO cells that are unable to synthesize GPI anchors (and hence lack GPI-anchored proteins) and their wild-type counterparts exhibit similar sensitivity to VacA-induced vacuolation (Schraw *et al*., 2002). Nonetheless, removal of GPI-anchored proteins from the cell surface reduced the ability of VacA to form channels in the plasma membrane as determined by measuring chloride flux (Gauthier *et al*., 2004).

## Internalization

After incubation of VacA with cells at 37˚C for several hours, VacA can be detected inside cells by a variety of immunological techniques (Garner and Cover, 1996; Sommi *et al*., 1998; Fiocca *et al*., 1999; Schraw *et al*., 2002; Li *et al*., 2004b), and the toxin becomes resistant to exogenously added proteases (McClain *et al*., 2000; Patel *et al*., 2002). Evidence implicates an active cellular process in the internalization of activated VacA, as internalization does not occur at 4˚C and is inhibited by depleting cells of ATP (McClain *et al*., 2000). Furthermore, VacA does not become protease resistant when incubated with rabbit erythrocytes, suggesting that VacA internalization does not occur spontaneously after binding to membranes (McClain *et al*., 2000). Once internalized, the 88-kDa protein persists inside the cell for hours with little noticeable degradation (Sommi *et al*., 1998; McClain *et al*., 2000). Multiple lines of evidence indicate that internalization of the toxin is required for many of the effects of VacA. Several studies have shown that transfecting the *vacA* gene into cells results in vacuolation, cytochrome c release, and apoptosis (de Bernard *et al*., 1997; Ye *et al*., 1999; Galmiche *et al*., 2000), suggesting that these activities result from actions of VacA at intracellular sites.

The cellular pathways by which VacA is endocytosed have not yet been clearly delineated. Two well-studied pathways utilized by cells to internalize ligands involve clathrin-coated pits or caveolae (Pelkmans and Helenius, 2003). VacA-induced vacuolation was observed among cells transfected with inhibitors of clathrin-mediated endocytosis (dominant-negative Eps15, or GFP-linked five tandem SH3 domains of intersectin) (Ricci *et al*., 2000), suggesting that VacA can be internalized via a clathrin-independent process. In support of this conclusion, cells treated with chlorpromazine, which inhibits assembly of clathrin-coated pits, exhibited an increase in vacuolation in response to VacA (Patel *et al*., 2002). Similarly, VacA appears to be internalized via a caveolae-independent process, as filipin blocks caveolae-mediated endocytosis (Orlandi and Fishman, 1998; Norkin *et al*., 2001), but did not affect VacA-mediated vacuolation (Patel *et al*., 2002). Dynamin, originally viewed as a protein involved in the formation of endosomes from the plasma membrane but subsequently implicated in a variety of steps involving endocytic trafficking inside cells (Kirchhausen, 1998; Schmid *et al*., 1998), has been shown to be required for VacA-induced vacuolation, but is not required for VacA internalization (Suzuki *et al*., 2001). Taken together, these results suggest that VacA is internalized by a dynamin-independent macropinocytotic pathway (Pelkmans and Helenius, 2003).

### Interactions with intracellular targets

Using VacA as bait in yeast two-hybrid screening of host cell libraries, two groups identified proteins that interact with VacA. One such protein is VacA-interacting protein of 54 kDa (VIP54) (de Bernard *et al*., 2000). VIP54 was isolated from a HeLa cell cDNA library, using a two-hybrid screen, as a protein that interacted with the amino-terminal portion of the VacA p55 domain. To validate the yeast two-hybrid results, a recombinant GST-tagged VIP54 was shown to interact with purified VacA. Confocal immunofluorescence microscopy with anti-VIP54 affinity-purified antibodies showed a fibrous pattern, suggesting that VIP54 associates with intermediate filaments. In support of this conclusion, immunofluorescence microscopy and immunoprecipitations indicated that VIP54 interacts with vimentin (either directly or indirectly). VIP54 is expressed in cultured cells and many tissues, including brain, muscle, kidney, and liver. Little is known at this time about VIP54, but the amino acid sequence of VIP54 is highly similar to SPIN90 (implicated in maintenance or assembly of the cytoskeleton in cardiac myocytes) (Lim *et al*., 2001) and to AF3p21 (found only in the nucleus) (Hayakawa *et al*., 2001). Potentially, these three proteins may represent alternate splice variants transcribed from a single gene. The functional significance of potential interactions between VacA and VIP54 is unknown.

An interaction between VacA and RACK1 was found using a yeast two-hybrid screen of a human gastric mucosa cDNA library (Hennig et al., 2001). The VacA fragment found to interact with RACK-1 consisted of the carboxy-terminal portion of the p33 domain and six residues from the p55 domain (amino acids 170–317). The interaction was confirmed by demonstrating an interaction in vitro between a recombinant RACK-1 and recombinant GST-tagged VacA. RACK1, a 36-kDa homologue of the b subunit of G proteins, belongs to a large family of regulatory proteins made up of highly conserved repeating WD40 units (Neer et al., 1994). RACK1 is composed of seven such WD-repeats. The cDNA inserts of two-hybrid positive clones encoded for the three carboxy-terminal WD motifs of RACK1 protein, suggesting that they are sufficient for interaction with VacA (Hennig et al., 2001). WD-repeats are involved in protein-protein interactions (Yaffe and Smerdon, 2004), and RACK1 has been reported to interact with a variety of cellular proteins (McCahill et al., 2002). The functional significance of potential interactions between VacA and RACK1 is unknown.

## VacA AS A VACCINE ANTIGEN

Oral immunization of mice with purified VacA protects mice from subsequent infection with *H. pylori* (Marchetti *et al*., 1995; Marchetti *et al*., 1998). Immunization with purified VacA conferred protection against subsequent challenge by *H. pylori* strains that produced vacuolating toxin activity *in vitro*, but such an immunization failed to protect against challenge with a wild-type strain that lacked detectable toxic activity for HeLa cells (Marchetti *et al*., 1995; Marchetti *et al*., 1998). One study reported that eradication of VacA-producing *H. pylori* from chronically infected mice can be achieved by therapeutic vaccination with VacA, and mice from which *H. pylori* were eradicated by the therapeutic vaccination were protected from subsequent challenge (Ghiara *et al*., 1997).

Many challenges remain in the development of an effective vaccine against *H. pylori. H. pylori* infection persists in the absence of antibiotic treatment, even though antibodies against numerous *H. pylori* antigens (including VacA) may be found in serum and gastric juice from the majority of *H. pylori*-infected humans (Cover *et al*., 1992a; Cover *et al*., 1993a; Perez-Perez *et al*., 1999). Thus, the immune response to VacA that is elicited in humans in response to *H. pylori* infection does not lead to clearance of the infection. Whether immune responses to natural *H. pylori* infection confer some measure of protection against subsequent *H. pylori* infection has not been investigated in detail. In animal studies, the failure of an s1-m1 VacA antigen to induce protective immunity against a non-toxigenic strain is likely due to antigenic diversity among different VacA proteins. In particular, it seems clear that many antibodies that are reactive with s1-m1 VacA fail to recognize s2-m2 VacA and may even fail to recognize s1-m1 from other *H. pylori* isolates (Vinion-Dubiel *et al*., 2001). Therefore, a vaccine based on multiple *H. pylori* antigens, including VacA, is likely to prove more effective than a vaccine based solely on a single VacA protein (Ghiara *et al*., 1997; Rossi *et al*., 2004).

## CONCLUSION

The activities attributed to VacA are numerous, and additional activities are likely to be discovered as researchers continue to investigate this toxin. Several VacA activities, including vacuole formation and effects on mitochondria, may be attributed to the channel-forming properties of VacA. In this sense, VacA resembles a pore-forming toxin that acts on intracellu-

lar targets (e.g., late endosomes and lysosomes, or mitochondria). Interestingly, there are numerous VacA proteins (i.e., s2-type proteins) for which *in vitro* activities have not been identified. Further studies of s2-type proteins may lead to the identification of activities shared with s1-type VacA toxins that are highly relevant *in vivo*. Alternatively, the s1- and s2-type VacA proteins may have evolved divergent functions suited to the distinct lifestyles of different *H. pylori* isolates. Further investigations of VacA are likely to lead to findings that are relevant for diverse fields of biology, and should also provide important insights into how *H. pylori* interacts with the human stomach.

## ACKNOWLEDGMENTS

Supported in part by the Vanderbilt University Medical Center Intramural Discovery Grant Program (MSM), and by the National Institutes of Health (RO1 AI39657 and DK53623) and the Medical Research Service of the Department of Veterans Affairs (TLC).

## REFERENCES

Adrian, M., Cover, T.L., Dubochet, J. and Heuser, J.E. (2002). Multiple oligomeric states of the *Helicobacter pylori* vacuolating toxin demonstrated by cryo-electron microscopy. *J. Mol. Biol*. **318,** 121–133.

Al-Awqati, Q. (1986). Proton-translocating ATPases. *Annu. Rev. Cell Biol*. **2,** 179–199.

Al-Awqati, Q. (1995). Chloride channels of intracellular organelles. *Curr. Opin. Cell Biol*. **7,** 504–508.

Alm, R.A., Ling, L.S., Moir, D.T., King, B.L., Brown, E.D., Doig, P.C., Smith, D.R., Noonan, B., Guild, B.C., deJonge, B.L., Carmel, G., Tummino, P.J., Caruso, A., Uria-Nickelsen, M., Mills, D.M., Ives, C., Gibson, R., Merberg, D., Mills, S.D., Jiang, Q., Taylor, D.E., Vovis, G.F. and Trust, T.J. (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori. Nature* **397,** 176–180.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol*. **215,** 403–410.

Argent, R.H., McGarr, C. and Atherton, J.C. (2004). Brefeldin A enhances *Helicobacter pylori* vacuolating cytotoxin-induced vacuolation of epithelial cells. *FEMS Microbiol. Lett*. **237,** 163–170.

Arselin, G., Giraud, M.F., Dautant, A., Vaillier, J., Brethes, D., Coulary-Salin, B., Schaeffer, J. and Velours, J. (2003). The GxxxG motif of the transmembrane domain of subunit e is involved in the dimerization/oligomerization of the yeast ATP synthase complex in the mitochondrial membrane. *Eur. J. Biochem*. **270,** 1875–1884.

Asahi, M., Tanaka, Y., Izumi, T., Ito, Y., Naiki, H., Kersulyte, D., Tsujikawa, K., Saito, M., Sada, K., Yanagi, S., Fujikawa, A., Noda, M. and Itokawa, Y. (2003). *Helicobacter pylori* CagA containing ITAM-like sequences localized to lipid rafts negatively regulates VacA-induced signaling *in vivo. Helicobacter* **8,** 1–14.

Ashorn, M., Cantet, F., Mayo, K. and Megraud, F. (2000). Cytoskeletal rearrangements induced by *Helicobacter pylori* strains in epithelial cell culture: possible role of the cytotoxin. *Dig. Dis. Sci*. **45,** 1774–1780.

Asundi, V.K. and Carey, D.J. (1995). Self-association of N-syndecan (syndecan-3) core protein is mediated by a novel structural motif in the transmembrane domain and ectodomain flanking region. *J. Biol. Chem*. **270,** 26404–26410.

Atherton, J.C., Cao, P., Peek, R.M., Jr., Tummuru, M.K., Blaser, M.J. and Cover, T.L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J. Biol. Chem*. **270,** 17771–17777.

Atherton, J.C., Cover, T.L., Papini, E. and Telford, J.L. (2001). In: *Helicobacter pylori: Physiology and Genetics* (eds. H.L.T. Mobley, G.L. Mendz, and S.L. Hazell), pp. 97–110. ASM Press, Washington, D.C.

Atherton, J.C., Sharp, P.M., Cover, T.L., Gonzalez-Valencia, G., Peek, R.M., Jr., Thompson, S.A., Hawkey, C.J. and Blaser, M.J. (1999). Vacuolating cytotoxin (*vacA*) alleles of *Helicobacter pylori* comprise two geographically widespread types, m1 and m2, and have evolved through limited recombination. *Curr. Microbiol*. **39,** 211–218.

Aviles-Jimenez, F., Letley, D.P., Gonzalez-Valencia, G., Salama, N., Torres, J. and Atherton, J.C. (2004). Evolution of the *Helicobacter pylori* vacuolating cytotoxin in a human stomach. *J. Bacteriol*. **186,** 5182–5185.

Ayraud, S., Janvier, B. and Fauchere, J.L. (2002). Experimental colonization of mice by fresh clinical isolates of *Helicobacter pylori* is not influenced by the *cagA* status and the *vacA* genotype. *FEMS Immunol. Med. Microbiol*. **34,** 169–172.

Barnard, F.M., Loughlin, M.F., Fainberg, H.P., Messenger, M.P., Ussery, D.W., Williams, P. and Jenks, P.J. (2004). Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori. Mol. Microbiol*. **51,** 15–32.

Beltran, P.J. and Bixby, J.L. (2003). Receptor protein tyrosine phosphatases as mediators of cellular adhesion. *Front. Biosci*. **8,** d87–99.

Benz, I. and Schmidt, M.A. (1992). AIDA-I, the adhesin involved in diffuse adherence of the diarrheagenic *Escherichia coli* strain 2787 (O126:H27), is synthesized via a precursor molecule. *Mol. Microbiol*. **6,** 1539–1546.

Boncristiano, M., Paccani, S.R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., Amedei, A., D'Elios, M.M., Telford, J.L. and Baldari, C.T. (2003). The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *J. Exp. Med*. **198,** 1887–1897.

Bonifacino, J.S. and Glick, B.S. (2004). The mechanisms of vesicle budding and fusion. *Cell* **116,** 153–166.

Brosig, B. and Langosch, D. (1998). The dimerization motif of the glycophorin A transmembrane segment in membranes: importance of glycine residues. *Protein Sci*. **7,** 1052–1056.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J. and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11,** 467–480.

Bumann, D., Aksu, S., Wendland, M., Janek, K., Zimny-Arndt, U., Sabarth, N., Meyer, T.F. and Jungblut, P.R. (2002). Proteome analysis of secreted proteins of the gastric pathogen *Helicobacter pylori. Infect. Immun*. **70,** 3396–3403.

Cantalupo, G., Alifano, P., Roberti, V., Bruni, C.B. and Bucci, C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. *EMBO J*. **20,** 683–693.

Caplan, S., Hartnell, L.M., Aguilar, R.C., Naslavsky, N. and Bonifacino, J.S. (2001). Human Vam6p promotes lysosome clustering and fusion *in vivo. J. Cell Biol*. **154,** 109–122.

Caputo, R., Tuccillo, C., Manzo, B.A., Zarrilli, R., Tortora, G., Blanco Cdel, V., Ricci, V., Ciardiello, F. and Romano, M. (2003). *Helicobacter pylori* VacA toxin up-regulates vascular endothelial growth factor expression in MKN 28 gastric cells through an epidermal growth factor receptor-, cyclooxygenase-2-dependent mechanism. *Clin. Cancer Res*. **9,** 2015–2021.

Carl, M., Dobson, M.E., Ching, W.M. and Dasch, G.A. (1990). Characterization of the gene encoding the protective paracrystalline-surface-layer protein of *Rickettsia prowazekii*: presence of a truncated identical homolog in *Rickettsia typhi*. *Proc. Natl. Acad. Sci. USA* **87,** 8237–8241.

Catrenich, C.E. and Chestnut, M.H. (1992). Character and origin of vacuoles induced in mammalian cells by the cytotoxin of *Helicobacter pylori*. *J. Med. Microbiol*. **37,** 389–395.

Chang, H.Y. and Yang, X. (2000). Proteases for cell suicide: functions and regulation of caspases. *Microbiol. Mol. Biol. Rev*. **64,** 821–846.

Cho, S.J., Kang, N.S., Park, S.Y., Kim, B.O., Rhee, D.K. and Pyo, S. (2003). Induction of apoptosis and expression of apoptosis related genes in human epithelial carcinoma cells by *Helicobacter pylori* VacA toxin. *Toxicon* **42,** 601–611.

Cover, T.L., Berg, D.E., Blaser, M.J. and Mobley, H.L.T. (2001). *H. pylori* pathogenesis. In: *Principles of bacterial Pathogenesis*, (ed. E.A. Groisman), pp. 510–558. Academic Press, San Diego.

Cover, T.L. and Blaser, M.J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem*. **267,** 10570–10575.

Cover, T.L., Cao, P., Lind, C.D., Tham, K.T. and Blaser, M.J. (1993a). Correlation between vacuolating cytotoxin production by *Helicobacter pylori* isolates *in vitro* and *in vivo*. *Infect. Immun*. **61,** 5008–5012.

Cover, T.L., Cao, P., Murthy, U.K., Sipple, M.S. and Blaser, M.J. (1992a). Serum neutralizing antibody response to the vacuolating cytotoxin of *Helicobacter pylori*. *J. Clin. Invest*. **90,** 913–918.

Cover, T.L., Halter, S.A. and Blaser, M.J. (1992b). Characterization of HeLa cell vacuoles induced by *Helicobacter pylori* broth culture supernatant. *Human Pathology* **23,** 1004–1010.

Cover, T.L., Hanson, P.I. and Heuser, J.E. (1997). Acid-induced dissociation of VacA, the *Helicobacter pylori* vacuolating cytotoxin, reveals its pattern of assembly. *J. Cell Biol*. **138,** 759–769.

Cover, T.L., Krishna, U.S., Israel, D.A. and Peek, R.M., Jr. (2003). Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Res*. **63,** 951–957.

Cover, T.L., Puryear, W., Pérez-Pérez, G.I. and Blaser, M.J. (1991). Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. *Infect. Immun*. **59,** 1264–1270.

Cover, T.L., Reddy, L.Y. and Blaser, M.J. (1993b). Effects of ATPase inhibitors on the response of HeLa cells to *Helicobacter pylori* vacuolating toxin. *Infect. Immun*. **61,** 1427–1431.

Cover, T.L., Tummuru, M.K.R., Cao, P., Thompson, S.A. and Blaser, M.J. (1994). Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J. Biol. Chem*. **269,** 10566–10573.

Cover, T.L., Vaughn, S.G., Cao, P. and Blaser, M.J. (1992c). Potentiation of *Helicobacter pylori* vacuolating toxin activity by nicotine and other weak bases. *J. Infect. Diseases* **166,** 1073–1078.

Czajkowsky, D.M., Iwamoto, H., Cover, T.L. and Shao, Z. (1999). The vacuolating toxin from *Helicobacter pylori* forms hexameric pores in lipid bilayers at low pH. *Proc. Natl. Acad. Sci. USA* **96,** 2001–2006.

Dailidiene, D., Dailide, G., Ogura, K., Zhang, M., Mukhopadhyay, A.K., Eaton, K.A., Cattoli, G., Kusters, J.G. and Berg, D.E. (2004). *Helicobacter acinonychis*: genetic and rodent infection studies of a *Helicobacter pylori*-like gastric pathogen of cheetahs and other big cats. *J. Bacteriol*. **186,** 356–365.

de Bernard, M., Arico, B., Papini, E., Rizzuto, R., Grandi, G., Rappuoli, R. and Montecucco, C. (1997). *Helicobacter pylori* toxin VacA induces vacuole formation by acting in the cell cytosol. *Mol. Microbiol*. **26,** 665–674.

de Bernard, M., Burroni, D., Papini, E., Rappuoli, R., Telford, J. and Montecucco, C. (1998a). Identification of the *Helicobacter pylori* VacA toxin domain active in the cell cytosol. *Infect. Immun*. **66,** 6014–6016.

de Bernard, M., Cappon, A., Pancotto, L., Ruggiero, P., Rivera, J., Del Giudice, G. and Montecucco, C. (2004). The *Helicobacter pylori* VacA cytotoxin activates RBL-2H3 cells by inducing cytosolic calcium oscillations. *Cell. Microbiol*. **7**, 191–198.

de Bernard, M., Moschioni, M., Habermann, A., Griffiths, G. and Montecucco, C. (2002). Cell vacuolization induced by *Helicobacter pylori* VacA cytotoxin does not depend on late endosomal SNAREs. *Cell. Microbiol*. **4,** 11–18.

de Bernard, M., Moschioni, M., Napolitani, G., Rappuoli, R. and Montecucco, C. (2000). The VacA toxin of *Helicobacter pylori* identifies a new intermediate filament-interacting protein. *EMBO J*. **19,** 48–56.

de Bernard, M., Moschioni, M., Papini, E., Telford, J., Rappuoli, R. and Montecucco, C. (1998b). Cell vacuolization induced by *Helicobacter pylori* VacA toxin: cell line sensitivity and quantitative estimation. *Toxicol. Lett*. **99,** 109–115.

de Bernard, M., Moschioni, M., Papini, E., Telford, J.L., Rappuoli, R. and Montecucco, C. (1998c). TPA and butyrate increase cell sensitivity to the vacuolating toxin of *Helicobacter pylori*. *FEBS Lett*. **436,** 218–222.

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., Fontana, A., Rappuoli, R. and Montecucco, C. (1995). Low pH activates the vacuolating toxin of *Helicobacter pylori*, which becomes acid and pepsin resistant. *J. Biol. Chem*. **270,** 23937–23940.

De Gusmao, V.R., Nogueira Mendes, E., De Magalhaes Queiroz, D.M., Aguiar Rocha, G., Camargos Rocha, A.M., Ramadan Ashour, A.A. and Teles Carvalho, A.S. (2000). *vacA* genotypes in *Helicobacter pylori* strains isolated from children with and without duodenal ulcer in Brazil. *J. Clin. Microbiol*. **38,** 2853–2857.

Debellis, L., Papini, E., Caroppo, R., Montecucco, C. and Curci, S. (2001). *Helicobacter pylori* cytotoxin VacA increases alkaline secretion in gastric epithelial cells. *Am. J. Physiol*. **281,** G1440–1448.

Desvaux, M., Parham, N.J. and Henderson, I.R. (2004). The autotransporter secretion system. *Res. Microbiol*. **155,** 53–60.

Dunn, B.E., Cohen, H. and Blaser, M.J. (1997). *Helicobacter pylori*. *Clin. Microbiol. Rev*. **10,** 720–741.

Duret, L., Gasteiger, E. and Perriere, G. (1996). LALNVIEW: a graphical viewer for pairwise sequence alignments. *Comput. Appl. Biosci*. **12,** 507–510.

Eaton, K.A., Cover, T.L., Tummuru, M.K., Blaser, M.J. and Krakowka, S. (1997). Role of vacuolating cytotoxin in gastritis due to *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun*. **65,** 3462–3464.

Everhart, J.E. (2000). Recent developments in the epidemiology of *Helicobacter pylori*. *Gastroenterol. Clin. North Am*. **29,** 559–578.

Feng, S., Hodzic, E., Kendall, L.V., Smith, A., Freet, K. and Barthold, S.W. (2002). Cloning and expression of a *Helicobacter bilis* immunoreactive protein. *Clin. Diagn. Lab. Immunol*. **9,** 627–632.

Ferrari, G., Langen, H., Naito, M. and Pieters, J. (1999). A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* **97,** 435–447.

Figueiredo, C., Machado, J.C., Pharoah, P., Seruca, R., Sousa, S., Carvalho, R., Capelinha, A.F., Quint, W., Caldas, C., van Doorn, L.J., Carneiro, F. and Sobrinho-Simoes, M. (2002). *Helicobacter*

*pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J. Natl. Cancer. Inst*. **94,** 1680–1687.

Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T.L. and Solcia, E. (1999). Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *J. Pathol*. **188,** 220–226.

Fiocca, R., Villani, L., Luinetti, O., Gianatti, A., Perego, M., Alvisi, C., Turpini, F. and Solcia, E. (1992). *Helicobacter* colonization and histopathological profile of chronic gastritis in patients with or without dyspepsia, mucosal erosion, and peptic ulcer: a morphological approach to the study of ulcerogenesis in man. *Virchows Arch. A Pathol. Anat. Histopathol*. **420,** 489–498.

Fischer, W., Buhrdorf, R., Gerland, E. and Haas, R. (2001). Outer membrane targeting of passenger proteins by the vacuolating cytotoxin autotransporter of *Helicobacter pylori*. *Infect. Immun*. **69,** 6769–6775.

Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P.E., Parton, R.G. and van der Goot, F.G. (2002). Differential sorting and fate of endocytosed GPI-anchored proteins. *EMBO J*. **21,** 3989–4000.

Forsyth, M.H., Atherton, J.C., Blaser, M.J. and Cover, T.L. (1998). Heterogeneity in levels of vacuolating cytotoxin gene (*vacA*) transcription among *Helicobacter pylori strains. Infect. Immun*. **66,** 3088–3094.

Forsyth, M.H. and Cover, T.L. (1999). Mutational analysis of the *vacA* promoter provides insight into gene transcription in *Helicobacter pylori*. *J. Bacteriol*. **181,** 2261–2266.

Fujikawa, A., Shirasaka, D., Yamamoto, S., Ota, H., Yahiro, K., Fukada, M., Shintani, T., Wada, A., Aoyama, N., Hirayama, T., Fukamachi, H. and Noda, M. (2003). Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. *Nat. Genet*. **33,** 375–381.

Galmiche, A., Rassow, J., Doye, A., Cagnol, S., Chambard, J.C., Contamin, S., de Thillot, V., Just, I., Ricci, V., Solcia, E., Van Obberghen, E. and Boquet, P. (2000). The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J*. **19,** 6361–6370.

Garner, J.A. and Cover, T.L. (1996). Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infect. Immun*. **64,** 4197–4203.

Gauthier, N.C., Ricci, V., Gounon, P., Doye, A., Tauc, M., Poujeol, P. and Boquet, P. (2004). Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the *Helicobacter pylori* vacuolating cytotoxin VacA in HeLa cells. *J. Biol. Chem*. **279,** 9481–9489.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R. and Haas, R. (2003). *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301,** 1099–1102.

Geisse, N.A., Cover, T.L., Henderson, R.M. and Edwardson, J.M. (2004). Targeting of *Helicobacter pylori* vacuolating toxin to lipid raft membrane domains analyzed by atomic force microscopy. *Biochem. J*. **381,** 911–917.

Gerhard, M., Lehn, N., Neumayer, N., Boren, T., Rad, R., Schepp, W., Miehlke, S., Classen, M. and Prinz, C. (1999). Clinical relevance of the *Helicobacter pylori* gene for blood-group, antigen-binding adhesin. *Proc. Natl. Acad. Sci. USA* **96,** 12778–12783.

Ghiara, P., Rossi, M., Marchetti, M., Di Tommaso, A., Vindigni, C., Ciampolini, F., Covacci, A., Telford, J.L., De Magistris, M.T., Pizza, M., Rappuoli, R. and Del Giudice, G. (1997). Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradicates an otherwise chronic infection and confers protection against reinfection. *Infect. Immun*. **65,** 4996–5002.

Gilmore, R.D., Jr., Joste, N. and McDonald, G.A. (1989). Cloning, expression, and sequence analysis of the gene encoding the 120 kD surface-exposed protein of *Rickettsia rickettsii*. *Mol. Microbiol*. **3,** 1579–1586.

Han, S.R., Schreiber, H.J., Bhakdi, S., Loos, M. and Maeurer, M.J. (1998). *vacA* genotypes and genetic diversity in clinical isolates of *Helicobacter pylori*. *Clin. Diagn. Lab. Immunol*. **5,** 139–145.

Hayakawa, A., Matsuda, Y., Daibata, M., Nakamura, H. and Sano, K. (2001). Genomic organization, tissue expression, and cellular localization of AF3p21, a fusion partner of MLL in therapy-related leukemia. *Genes Chromosomes Cancer* **30,** 364–374.

Hebert, T.E., Moffett, S., Morello, J.P., Loisel, T.P., Bichet, D.G., Barret, C. and Bouvier, M. (1996). A peptide derived from a $\beta_2$-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J. Biol. Chem*. **271,** 16384–16392.

Henderson, I.R., Navarro-Garcia, F. and Nataro, J.P. (1998). The great escape: structure and function of the autotransporter proteins. *Trends Microbiol*. **6,** 370–378.

Henikoff, S., Henikoff, J.G., Alford, W.J. and Pietrokovski, S. (1995). Automated construction and graphical presentation of protein blocks from unaligned sequences. *Gene* **163,** GC17–26.

Hennig, E.E., Butruk, E. and Ostrowski, J. (2001). RACK1 protein interacts with *Helicobacter pylori* VacA cytotoxin: the yeast two-hybrid approach. *Biochem. Biophys. Res. Commun*. **289,** 103–110.

Hiltbold, E.M. and Roche, P.A. (2002). Trafficking of MHC class II molecules in the late secretory pathway. *Curr. Opin. Immunol*. **14,** 30–35.

Hotchin, N.A., Cover, T.L. and Akhtar, N. (2000). Cell vacuolation induced by the VacA cytotoxin of *Helicobacter pylori* is regulated by the Rac1 GTPase. *J. Biol. Chem*. **275,** 14009–14012.

Huang, X. and Miller, W. (1991). A time-efficient, linear-space local similarity algorithm. *Adv. Appl. Math*. **12,** 337–357.

Ikonomov, O.C., Sbrissa, D., Yoshimori, T., Cover, T.L. and Shisheva, A. (2002). PIKfyve Kinase and SKD1 AAA ATPase define distinct endocytic compartments. Only PIKfyve expression inhibits the cell-vacoulating activity of *Helicobacter pylori* VacA toxin. *J. Biol. Chem*. **277,** 46785–46790.

Ilver, D., Barone, S., Mercati, D., Lupetti, P. and Telford, J.L. (2004). *Helicobacter pylori* toxin VacA is transferred to host cells via a novel contact-dependent mechanism. *Cell. Microbiol*. **6,** 167–174.

Ito, Y., Azuma, T., Ito, S., Suto, H., Miyaji, H., Yamazaki, Y., Kohli, Y. and Kuriyama, M. (1998). Full-length sequence analysis of the *vacA* gene from cytotoxic and noncytotoxic *Helicobacter pylori*. *J. Infect. Dis*. **178,** 1391–1398.

Iwamoto, H., Czajkowsky, D.M., Cover, T.L., Szabo, G. and Shao, Z. (1999). VacA from *Helicobacter pylori*: a hexameric chloride channel. *FEBS Lett*. **450,** 101–104.

Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J.C., Reyrat, J.M., Rappuoli, R. and Telford, J.L. (2000). Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infect. Immun*. **68,** 3754–3757.

Ji, X., Frati, F., Barone, S., Pagliaccia, C., Burroni, D., Xu, G., Rappuoli, R., Reyrat, J.M. and Telford, J.L. (2002). Evolution of functional polymorphism in the gene coding for the *Helicobacter pylori* cytotoxin. *FEMS Microbiol. Lett*. **206,** 253–258.

Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R. and Bagshaw, P. (2000). A role for the bacterial outer membrane in the pathogenesis of *Helicobacter pylori* infection. *FEMS Microbiol. Lett*. **182,** 259–264.

Kidd, M., Lastovica, A.J., Atherton, J.C. and Louw, J.A. (1999). Heterogeneity in the *Helicobacter pylori vacA* and *cagA* genes: association with gastroduodenal disease in South Africa? *Gut* **45,** 499–502.

Kim, S., Chamberlain, A.K. and Bowie, J.U. (2004). Membrane channel structure of *Helicobacter pylori* vacuolating toxin: role of

multiple GXXXG motifs in cylindrical channels. *Proc. Natl. Acad. Sci. USA* **101,** 5988–5991.

Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hirayama, T. and Kondo, T. (1999). Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb. Pathog*. **26,** 45–52.

Kirchhausen, T. (1998). Vesicle formation: dynamic dynamin lives up to its name. *Curr. Biol*. **8,** R792–794.

Kleiger, G., Grothe, R., Mallick, P. and Eisenberg, D. (2002). GXXXG and AXXXA: common α-helical interaction motifs in proteins, particularly in extremophiles. *Biochemistry* **41,** 5990–5997.

Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P.H., Stremmel, W. and Rudi, J. (2001). Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. *Infect. Immun*. **69,** 5080–5087.

Kuo, C.H. and Wang, W.C. (2003). Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells. *Biochem. Biophys. Res. Commun*. **303,** 640–644.

Lanzavecchia, S., Bellon, P.L., Lupetti, P., Dallai, R., Rappuoli, R. and Telford, J.L. (1998). Three-dimensional reconstruction of metal replicas of the *Helicobacter pylori* vacuolating cytotoxin. *J. Struct. Biol*. **121,** 9–18.

Lebrand, C., Corti, M., Goodson, H., Cosson, P., Cavalli, V., Mayran, N., Faure, J. and Gruenberg, J. (2002). Late endosome motility depends on lipids via the small GTPase Rab7. *EMBO J*. **21,** 1289–1300.

Lee, S.F., Shah, S., Yu, C., Wigley, W.C., Li, H., Lim, M., Pedersen, K., Han, W., Thomas, P., Lundkvist, J., Hao, Y.H. and Yu, G. (2004). A conserved GXXXG motif in APH-1 is critical for assembly and activity of the gamma-secretase complex. *J. Biol. Chem*. **279,** 4144–4152.

Lemmon, M.A., Flanagan, J.M., Treutlein, H.R., Zhang, J. and Engelman, D.M. (1992). Sequence specificity in the dimerization of transmembrane alpha-helices. *Biochemistry* **31,** 12719–12725.

Lemmon, M.A., Treutlein, H.R., Adams, P.D., Brunger, A.T. and Engelman, D.M. (1994). A dimerization motif for transmembrane alpha-helices. *Nat. Struct. Biol*. **1,** 157–163.

Letley, D.P. and Atherton, J.C. (2000). Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. *J. Bacteriol*. **182,** 3278–3280.

Letley, D.P., Lastovica, A., Louw, J.A., Hawkey, C.J. and Atherton, J.C. (1999). Allelic diversity of the *Helicobacter pylori* vacuolating cytotoxin gene in South Africa: rarity of the *vacA* s1a genotype and natural occurrence of an s2/m1 allele. *J. Clin. Microbiol*. **37,** 1203–1205.

Letley, D.P., Rhead, J.L., Twells, R.J., Dove, B. and Atherton, J.C. (2003). Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. *J. Biol. Chem*. **278,** 26734–26741.

Leunk, R.D., P.T., J., David, B.C., Kraft, W.G. and Morgan, D.R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J. Med. Microbiol*. **26,** 93–99.

Li, R., Gorelik, R., Nanda, V., Law, P.B., Lear, J.D., DeGrado, W.F. and Bennett, J.S. (2004a). Dimerization of the transmembrane domain of Integrin $\alpha_{IIb}$ subunit in cell membranes. *J. Biol. Chem*. **279,** 26666–26673.

Li, X., Wang, T., Zhao, Z. and Weinman, S.A. (2002). The ClC-3 chloride channel promotes acidification of lysosomes in CHO-K1 and Huh-7 cells. *Am. J. Physiol. Cell Physiol*. **282,** C1483–1491.

Li, Y., Wandinger-Ness, A., Goldenring, J.R. and Cover, T.L. (2004b). Clustering and redistribution of late endocytic compartments in response to *Helicobacter pylori* vacuolating toxin. *Mol. Biol. Cell* **15,** 1946–1959.

Lim, C.S., Park, E.S., Kim, D.J., Song, Y.H., Eom, S.H., Chun, J.S., Kim, J.H., Kim, J.K., Park, D. and Song, W.K. (2001). SPIN90 (SH3 protein interacting with Nck, 90 kDa), an adaptor protein that is developmentally regulated during cardiac myocyte differentiation. *J. Biol. Chem*. **276,** 12871–12878.

Lupetti, P., Heuser, J.E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P.L., Dallai, R., Rappuoli, R. and Telford, J.L. (1996). Oligomeric and subunit structure of the *Helicobacter pylori* vacuolating cytotoxin. *J. Cell Biol*. **133,** 801–807.

MacKenzie, K.R., Prestegard, J.H. and Engelman, D.M. (1997). A transmembrane helix dimer: structure and implications. *Science* **276,** 131–133.

Marchetti, M., Aricò, B., Burroni, D., Figura, N., Rappuoli, R. and Ghiara, P. (1995). Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* **267,** 1655–1658.

Marchetti, M., Rossi, M., Giannelli, V., Giuliani, M.M., Pizza, M., Censini, S., Covacci, A., Massari, P., Pagliaccia, C., Manetti, R., Telford, J.L., Douce, G., Dougan, G., Rappuoli, R. and Ghiara, P. (1998). Protection against *Helicobacter pylori* infection in mice by intragastric vaccination with *H. pylori* antigens is achieved using a non-toxic mutant of *E. coli* heat-labile enterotoxin (LT) as adjuvant. *Vaccine* **16,** 33–37.

Marshall, B.J. and Warren, J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1,** 1311–1315.

Massari, P., Manetti, R., Burroni, D., Nuti, S., Norais, N., Rappuoli, R. and Telford, J.L. (1998). Binding of the *Helicobacter pylori* vacuolating cytotoxin to target cells. *Infect. Immun*. **66,** 3981–3984.

McCahill, A., Warwicker, J., Bolger, G.B., Houslay, M.D. and Yarwood, S.J. (2002). The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. *Mol. Pharmacol*. **62,** 1261–1273.

McClain, M.S., Cao, P. and Cover, T.L. (2001a). Amino-terminal hydrophobic region of *Helicobacter pylori* vacuolating cytotoxin (VacA) mediates transmembrane protein dimerization. *Infect. Immun*. **69,** 1181–1184.

McClain, M.S., Cao, P., Iwamoto, H., Vinion-Dubiel, A.D., Szabo, G., Shao, Z. and Cover, T.L. (2001b). A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. *J. Bacteriol*. **183,** 6499–6508.

McClain, M.S., Iwamoto, H., Cao, P., Vinion-Dubiel, A.D., Li, Y., Szabo, G., Shao, Z. and Cover, T.L. (2003). Essential role of a GXXXG motif for membrane channel formation by *Helicobacter pylori* vacuolating toxin. *J. Biol. Chem*. **278,** 12101–12108.

McClain, M.S., Schraw, W., Ricci, V., Boquet, P. and Cover, T.L. (2000). Acid-activation of *Helicobacter pylori* vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells. *Mol. Microbiol*. **37,** 433–442.

Menaker, R.J., Ceponis, P.J. and Jones, N.L. (2004). *Helicobacter pylori* induces apoptosis of macrophages in association with alterations in the mitochondrial pathway. *Infect. Immun*. **72,** 2889–2898.

Mendrola, J.M., Berger, M.B., King, M.C. and Lemmon, M.A. (2002). The single transmembrane domains of ErbB receptors self-associate in cell membranes. *J. Biol. Chem*. **277,** 4704–4712.

Merrell, D.S., Thompson, L.J., Kim, C.C., Mitchell, H., Tompkins, L.S., Lee, A. and Falkow, S. (2003). Growth phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect. Immun*. **71,** 6510–6525.

Mohrmann, K. and van der Sluijs, P. (1999). Regulation of membrane transport through the endocytic pathway by rabGTPases. *Mol. Membr. Biol*. **16,** 81–87.

Molinari, M., Galli, C., de Bernard, M., Norais, N., Ruysschaert, J.M., Rappuoli, R. and Montecucco, C. (1998a). The acid activation of *Helicobacter pylori* toxin VacA: structural and membrane binding studies. *Biochem. Biophys. Res. Commun*. **248,** 334–340.

Molinari, M., Galli, C., Norais, N., Telford, J.L., Rappuoli, R., Luzio, J.P. and Montecucco, C. (1997). Vacuoles induced by *Helicobacter pylori* toxin contain both late endosomal and lysosomal markers. *J. Biol. Chem*. **272,** 25339–25344.

Molinari, M., Salio, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A. and Montecucco, C. (1998b). Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *J. Exp. Med*. **187,** 135–140.

Moll, G., Papini, E., Colonna, R., Burroni, D., Telford, J., Rappuoli, R. and Montecucco, C. (1995). Lipid interaction of the 37-kDa and 58-kDa fragments of the *Helicobacter pylori* cytotoxin. *Eur. J. Biochem*. **234,** 947–952.

Montecucco, C., Papini, E., de Bernard, M., Telford, J.L. and Rappuoli, R. (1999). *Helicobacter pylori* vacuolating cytotoxin and associated pathogenic factors. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), pp. 264–286. Academic Press, London.

Morbiato, L., Tombola, F., Campello, S., Del Giudice, G., Rappuoli, R., Zoratti, M. and Papini, E. (2001). Vacuolation induced by VacA toxin of *Helicobacter pylori* requires the intracellular accumulation of membrane permeant bases, $Cl^-$ and water. *FEBS Lett*. **508,** 479–483.

Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P. and Piper, R.C. (2000). Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and is required for late endosome-lysosome fusion. *Mol. Biol. Cell* **11,** 3137–3153.

Nakajima, S., Bamba, N. and Hattori, T. (2004). Histological aspects and role of mast cells in *Helicobacter pylori*-infected gastritis. *Aliment. Pharmacol. Ther*. **20 Suppl 1,** 165–170.

Nakajima, S., Krishnan, B., Ota, H., Segura, A.M., Hattori, T., Graham, D.Y. and Genta, R.M. (1997). Mast cell involvement in gastritis with or without *Helicobacter pylori* infection. *Gastroenterology* **113,** 746–754.

Nakamura, N., Hirata, A., Ohsumi, Y. and Wada, Y. (1997). Vam2/Vps41p and Vam6/Vps39p are components of a protein complex on the vacuolar membranes and involved in the vacuolar assembly in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem*. **272,** 11344–11349.

Nakamura, N., Yamamoto, A., Wada, Y. and Futai, M. (2000). Syntaxin 7 mediates endocytic trafficking to late endosomes. *J. Biol. Chem*. **275,** 6523–6529.

Nakayama, M., Kimura, M., Wada, A., Yahiro, K., Ogushi, K., Niidome, T., Fujikawa, A., Shirasaka, D., Aoyama, N., Kurazono, H., Noda, M., Moss, J. and Hirayama, T. (2004). *Helicobacter pylori* VacA activates the p38/activating transcription factor 2-mediated signal pathway in AZ-521 cells. *J. Biol. Chem*. **279,** 7024–7028.

Neer, E.J., Schmidt, C.J., Nambudripad, R. and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. *Nature* **371,** 297–300.

Nguyen, V.Q., Caprioli, R.M. and Cover, T.L. (2001). Carboxy-terminal proteolytic processing of *Helicobacter pylori* vacuolating toxin. *Infect. Immun*. **69,** 543–546.

Nieva, J.L. and Agirre, A. (2003). Are fusion peptides a good model to study viral cell fusion? *Biochim. Biophys. Acta* **1614,** 104–115.

Nogueira, C., Figueiredo, C., Carneiro, F., Gomes, A.T., Barreira, R., Figueira, P., Salgado, C., Belo, L., Peixoto, A., Bravo, J.C., Bravo, L.E., Realpe, J.L., Plaisier, A.P., Quint, W.G., Ruiz, B., Correa, P. and van Doorn, L.J. (2001). *Helicobacter pylori* genotypes may determine gastric histopathology. *Am. J. Pathol*. **158,** 647–654.

Norkin, L.C., Wolfrom, S.A. and Stuart, E.S. (2001). Association of caveolin with *Chlamydia trachomatis* inclusions at early and late stages of infection. *Exp. Cell Res*. **266,** 229–238.

Orlandi, P.A. and Fishman, P.H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol*. **141,** 905–915.

Padilla, P.I., Wada, A., Yahiro, K., Kimura, M., Niidome, T., Aoyagi, H., Kumatori, A., Anami, M., Hayashi, T., Fujisawa, J., Saito, H., Moss, J. and Hirayama, T. (2000). Morphologic differentiation of HL-60 cells is associated with appearance of RPTPβ and induction of *Helicobacter pylori* VacA sensitivity. *J. Biol. Chem*. **275,** 15200–15206.

Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X., Burroni, D., Cover, T.L., Papini, E., Rappuoli, R., Telford, J.L. and Reyrat, J.M. (1998). The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc. Natl. Acad. Sci. USA* **95,** 10212–10217.

Pai, R., Cover, T.L. and Tarnawski, A.S. (1999). *Helicobacter pylori* vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. *Biochem. Biophys. Res. Commun*. **262,** 245–250.

Pai, R., Sasaki, E. and Tarnawski, A.S. (2000). *Helicobacter pylori* vacuolating cytotoxin (VacA) alters cytoskeleton-associated proteins and interferes with re-epithelialization of wounded gastric epithelial monolayers. *Cell Biol. Int*. **24,** 291–301.

Pan, Z.J., Berg, D.E., van der Hulst, R.W., Su, W.W., Raudonikiene, A., Xiao, S.D., Dankert, J., Tytgat, G.N. and van der Ende, A. (1998). Prevalence of vacuolating cytotoxin production and distribution of distinct *vacA* alleles in *Helicobacter pylori* from China. *J. Infect. Dis*. **178,** 220–226.

Papini, E., Bugnoli, M., De Bernard, M., Figura, N., Rappuoli, R. and Montecucco, C. (1993). Bafilomycin A1 inhibits *Helicobacter pylori*-induced vacuolization of HeLa cells. *Mol. Microbiol*. **7,** 323–327.

Papini, E., de Bernard, M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R. and Montecucco, C. (1994). Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proc. Natl. Acad. Sci. USA* **91,** 9720–9724.

Papini, E., Gottardi, E., Satin, B., de Bernard, M., Massari, P., Telford, J., Rappuoli, R., Sato, S.B. and Montecucco, C. (1996). The vacuolar ATPase proton pump is present on intracellular vacuoles induced by *Helicobacter pylori*. *J. Med. Microbiol*. **45,** 84–89.

Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J.L., Manetti, R., Rappuoli, R., Zerial, M. and Montecucco, C. (1997). The small GTP binding protein rab7 is essential for cellular vacuolation induced by *Helicobacter pylori* cytotoxin. *EMBO J*. **16,** 15–24.

Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J.L., Rappuoli, R. and Montecucco, C. (1998). Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *J. Clin. Invest*. **102,** 813–820.

Papini, E., Zoratti, M. and Cover, T.L. (2001). In search of the *Helicobacter pylori* VacA mechanism of action. *Toxicon* **39,** 1757–1767.

Patel, H.K., Willhite, D.C., Patel, R.M., Ye, D., Williams, C.L., Torres, E.M., Marty, K.B., MacDonald, R.A. and Blanke, S.R. (2002). Plasma membrane cholesterol modulates cellular vacuolation induced by the *Helicobacter pylori* vacuolating cytotoxin. *Infect. Immun*. **70,** 4112–4123.

Peek, R.M., Jr., Blaser, M.J., Mays, D.J., Forsyth, M.H., Cover, T.L., Song, S.Y., Krishna, U. and Pietenpol, J.A. (1999). *Helicobacter pylori* strain-specific genotypes and modulation of the gastric epithelial cell cycle. *Cancer Res*. **59,** 6124–6131.

Pelicic, V., Reyrat, J.M., Sartori, L., Pagliaccia, C., Rappuoli, R., Telford, J.L., Montecucco, C. and Papini, E. (1999). *Helicobacter pylori* VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. *Microbiology* **145,** 2043–2050.

Pelkmans, L. and Helenius, A. (2003). Insider information: what viruses tell us about endocytosis. *Curr. Opin. Cell Biol*. **15,** 414–422.

Perez-Perez, G.I., Peek, R.M., Jr., Atherton, J.C., Blaser, M.J. and Cover, T.L. (1999). Detection of anti-VacA antibody responses in serum and gastric juice samples using type s1/m1 and s2/m2 *Helicobacter pylori* VacA antigens. *Clin. Diagn. Lab. Immunol*. **6,** 489–493.

Phadnis, S.H., Ilver, D., Janzon, L., Normark, S. and Westblom, T.U. (1994). Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. *Infect. Immun*. **62,** 1557–1565.

Pohlner, J., Halter, R., Beyreuther, K. and Meyer, T.F. (1987). Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325,** 458–462.

Polgar, O., Robey, R.W., Morisaki, K., Dean, M., Michejda, C., Sauna, Z.E., Ambudkar, S.V., Tarasova, N. and Bates, S.E. (2004). Mutational analysis of ABCG2: role of the GXXXG motif. *Biochemistry* **43,** 9448–9456.

Poulsen, K., Brandt, J., Hjorth, J.P., Thogersen, H.C. and Kilian, M. (1989). Cloning and sequencing of the immunoglobulin A1 protease gene (*iga*) of *Haemophilus influenzae* serotype b. *Infect. Immun*. **57,** 3097–3105.

Reyrat, J.M., Lanzavecchia, S., Lupetti, P., de Bernard, M., Pagliaccia, C., Pelicic, V., Charrel, M., Ulivieri, C., Norais, N., Ji, X., Cabiaux, V., Papini, E., Rappuoli, R. and Telford, J.L. (1999). 3D imaging of the 58 kDa cell binding subunit of the *Helicobacter pylori* cytotoxin. *J. Mol. Biol*. **290,** 459–470.

Ricci, V., Ciacci, C., Zarrilli, R., Sommi, P., Tummuru, M.K., Del Vecchio Blanco, C., Bruni, C.B., Cover, T.L., Blaser, M.J. and Romano, M. (1996). Effect of *Helicobacter pylori* on gastric epithelial cell migration and proliferation *in vitro*: role of VacA and CagA. *Infect. Immun*. **64,** 2829–2833.

Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E. and Boquet, P. (2000). High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol. Biol. Cell* **11,** 3897–3909.

Ricci, V., Sommi, P., Fiocca, R., Romano, M., Solcia, E. and Ventura, U. (1997). *Helicobacter pylori* vacuolating toxin accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolating activity of ammonia. *J. Pathol*. **183,** 453–459.

Rossi, G., Ruggiero, P., Peppoloni, S., Pancotto, L., Fortuna, D., Lauretti, L., Volpini, G., Mancianti, S., Corazza, M., Taccini, E., Di Pisa, F., Rappuoli, R. and Del Giudice, G. (2004). Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. *Infect. Immun*. **72,** 3252–3259.

Rudi, J., Kolb, C., Maiwald, M., Kuck, D., Sieg, A., Galle, P.R. and Stremmel, W. (1998). Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *J. Clin. Microbiol*. **36,** 944–948.

Russ, W.P. and Engelman, D.M. (1999). TOXCAT: a measure of transmembrane helix association in a biological membrane. *Proc. Natl. Acad. Sci. USA* **96,** 863–868.

Russ, W.P. and Engelman, D.M. (2000). The GxxxG motif: a framework for transmembrane helix-helix association. *J. Mol. Biol*. **296,** 911–919.

Salama, N.R., Otto, G., Tompkins, L. and Falkow, S. (2001). Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. *Infect. Immun*. **69,** 730–736.

Satin, B., Norais, N., Telford, J., Rappuoli, R., Murgia, M., Montecucco, C. and Papini, E. (1997). Effect of *Helicobacter pylori* vacuolating toxin on maturation and extracellular release of procathepsin D and on epidermal growth factor degradation. *J. Biol. Chem*. **272,** 25022–25028.

Schmid, S.L., McNiven, M.A. and De Camilli, P. (1998). Dynamin and its partners: a progress report. *Curr. Opin. Cell Biol*. **10,** 504–512.

Schmitt, W. and Haas, R. (1994). Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Mol. Microbiol*. **12,** 307–319.

Schraw, W., Li, Y., McClain, M.S., van der Goot, F.G. and Cover, T.L. (2002). Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. *J. Biol. Chem*. **277,** 34642–34650.

Seto, K., Hayashi-Kuwabara, Y., Yoneta, T., Suda, H. and Tamaki, H. (1998). Vacuolation induced by cytotoxin from *Helicobacter pylori* is mediated by the EGF receptor in HeLa cells. *FEBS Lett*. **431,** 347–350.

Sharom, F.J. and Lehto, M.T. (2002). Glycosylphosphatidylinositol-anchored proteins: structure, function, and cleavage by phosphatidylinositol-specific phospholipase C. *Biochem. Cell Biol*. **80,** 535–549.

Smoot, D.T., Resau, J.H., Earlington, M.H., Simpson, M. and Cover, T.L. (1996). Effects of *Helicobacter pylori* vacuolating cytotoxin on primary cultures of human gastric epithelial cells. *Gut* **39,** 795–799.

Sommi, P., Ricci, V., Fiocca, R., Necchi, V., Romano, M., Telford, J.L., Solcia, E. and Ventura, U. (1998). Persistence of *Helicobacter pylori* VacA toxin and vacuolating potential in cultured gastric epithelial cells. *Am. J. Physiol*. **275,** G681–688.

Sonawane, N.D., Thiagarajah, J.R. and Verkman, A.S. (2002). Chloride concentration in endosomes measured using a ratioable fluorescent $Cl^-$ indicator: evidence for chloride accumulation during acidification. *J. Biol. Chem*. **277,** 5506–5513.

Strobel, S., Bereswill, S., Balig, P., Allgaier, P., Sonntag, H.G. and Kist, M. (1998). Identification and analysis of a new *vacA* genotype variant of *Helicobacter pylori* in different patient groups in Germany. *J. Clin. Microbiol*. **36,** 1285–1289.

Struyvé, M., Moons, M. and Tommassen, J. (1991). Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. *J. Mol. Biol*. **218,** 141–148.

Suerbaum, S. and Michetti, P. (2002). *Helicobacter pylori* infection. *N. Engl. J. Med*. **347,** 1175–1186.

Suerbaum, S., Smith, J.M., Bapumia, K., Morelli, G., Smith, N.H., Kunstmann, E., Dyrek, I. and Achtman, M. (1998). Free recombination within *Helicobacter pylori*. *Proc. Natl. Acad. Sci. USA* **95,** 12619–12624.

Sundrud, M.S., Torres, V.J., Unutmaz, D. and Cover, T.L. (2004). Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc. Natl. Acad. Sci. USA* **101,** 7727–7732.

Supajatura, V., Ushio, H., Wada, A., Yahiro, K., Okumura, K., Ogawa, H., Hirayama, T. and Ra, C. (2002). Cutting edge: VacA, a vacuolating cytotoxin of *Helicobacter pylori*, directly activates mast cells for migration and production of proinflammatory cytokines. *J. Immunol*. **168,** 2603–2607.

Suzuki, J., Ohnishi, H., Shibata, H., Wada, A., Hirayama, T., Iiri, T., Ueda, N., Kanamaru, C., Tsuchida, T., Mashima, H., Yasuda, H. and Fujita, T. (2001). Dynamin is involved in human epithelial cell vacuolation caused by the *Helicobacter pylori*-produced cytotoxin VacA. *J. Clin. Invest*. **107,** 1329.

Suzuki, J., Ohnishi, H., Wada, A., Hirayama, T., Ohno, H., Ueda, N., Yasuda, H., Iiri, T., Wada, Y., Futai, M. and Mashima, H. (2003). Involvement of syntaxin 7 in human gastric epithelial cell vacuolation induced by the *Helicobacter pylori*-produced cytotoxin VacA. *J. Biol. Chem*. **278,** 25585–25590.

Szabo, I., Brutsche, S., Tombola, F., Moschioni, M., Satin, B., Telford, J.L., Rappuoli, R., Montecucco, C., Papini, E. and Zoratti, M.

(1999). Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO J*. **18,** 5517–5527.

Szczebara, F., Dhaenens, L., Armand, S. and Husson, M.O. (1999). Regulation of the transcription of genes encoding different virulence factors in *Helicobacter pylori* by free iron. *FEMS Microbiol. Lett*. **175,** 165–170.

Szewczyk, A. (1998). The intracellular potassium and chloride channels: properties, pharmacology, and function (review). *Mol. Membr. Biol*. **15,** 49–58.

Tabel, G., Hoa, N.T., Tarnawski, A., Chen, J., Domek, M. and Ma, T.Y. (2003). *Helicobacter pylori* infection inhibits healing of the wounded duodenal epithelium *in vitro. J. Lab. Clin. Med*. **142,** 421–430.

Telford, J.L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., Tecce, M.F., Censini, S., Covacci, A., Xiang, Z., Papini, E., Montecucco, C., Parente, L. and Rappuoli, R. (1994). Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J. Exp. Med*. **179,** 1653–1658.

Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. and Dixit, V.M. (1995). Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81,** 801–809.

Tomb, J.-F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E.F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H.G., Glodek, A., McKenney, K., Fitzegerald, L.M., Lee, N., Adams, M.D., Hickey, E.K., Berg, D.E., Gocayne, J.D., Utterback, T.R., Peterson, J.D., Kelley, J.M., Cotton, M.D., Weidman, J.M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W.S., Borodovsky, M., Karp, P.D., Smith, H.O., Fraser, C.M. and Venter, J.C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori. Nature* **388,** 539–547.

Tombola, F., Carlesso, C., Szabo, I., de Bernard, M., Reyrat, J.M., Telford, J.L., Rappuoli, R., Montecucco, C., Papini, E. and Zoratti, M. (1999a). *Helicobacter pylori* vacuolating toxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophys. J.* **76,** 1401–1409.

Tombola, F., Morbiato, L., Del Giudice, G., Rappuoli, R., Zoratti, M. and Papini, E. (2001). The *Helicobacter pylori* VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J. Clin. Invest*. **108,** 929–937.

Tombola, F., Oregna, F., Brutsche, S., Szabo, I., Del Giudice, G., Rappuoli, R., Montecucco, C., Papini, E. and Zoratti, M. (1999b). Inhibition of the vacuolating and anion channel activities of the VacA toxin of *Helicobacter pylori. FEBS Lett*. **460,** 221–225.

Torres, V.J., McClain, M.S. and Cover, T.L. (2004). Interactions between p-33 and p-55 domains of the *Helicobacter pylori* vacuolating cytotoxin (VacA). *J. Biol. Chem*. **279,** 2324–2331.

Tricottet, V., Bruneval, P., Vire, O., Camilleri, J.P., Bloch, F., Bonte, N. and Roge, J. (1986). *Campylobacter*-like organisms and surface epithelium abnormalities in active, chronic gastritis in humans: an ultrastructural study. *Ultrastruct. Pathol*. **10,** 113–122.

Utt, M., Danielsson, B. and Wadstrom, T. (2001). *Helicobacter pylori* vacuolating cytotoxin binding to a putative cell surface receptor, heparan sulfate, studied by surface plasmon resonance. *FEMS Immunol. Med. Microbiol*. **30,** 109–113.

van Amsterdam, K., van Vliet, A.H., Kusters, J.G., Feller, M., Dankert, J. and van der Ende, A. (2003). Induced *Helicobacter pylori* vacuolating cytotoxin VacA expression after initial colonization of human gastric epithelial cells. *FEMS Immunol. Med. Microbiol*. **39,** 251–256.

Van Doorn, L.J., Figueiredo, C., Megraud, F., Pena, S., Midolo, P., Queiroz, D.M., Carneiro, F., Vanderborght, B., Pegado, M.D., Sanna, R., De Boer, W., Schneeberger, P.M., Correa, P., Ng, E.K., Atherton, J., Blaser, M.J. and Quint, W.G. (1999). Geographic distribution of *vacA* allelic types of *Helicobacter pylori. Gastroenterology* **116,** 823–830.

van Doorn, L.J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E.K., Atherton, J.C., Blaser, M.J. and Quint, W.G. (1998). Expanding allelic diversity of *Helicobacter pylori vacA*. *J Clin Microbiol* **36,** 2597–2603.

van Doorn, N.E., Namavar, F., Sparrius, M., Stoof, J., van Rees, E.P., van Doorn, L.J. and Vandenbroucke-Grauls, C.M. (1999a). *Helicobacter pylori*-associated gastritis in mice is host and strain specific. *Infect. Immun*. **67,** 3040–3046.

van Doorn, N.E., van Rees, E.P., Namavar, F., Ghiara, P., Vandenbroucke-Grauls, C.M. and de Graaff, J. (1999b). The inflammatory response in CD1 mice shortly after infection with a CagA+/VacA+ *Helicobacter pylori* strain. *Clin. Exp. Immunol*. **115,** 421–427.

Vinion-Dubiel, A.D., McClain, M.S., Cao, P., Mernaugh, R.L. and Cover, T.L. (2001). Antigenic diversity among *Helicobacter pylori* vacuolating toxins. *Infect. Immun*. **69,** 4329–4336.

Vinion-Dubiel, A.D., McClain, M.S., Czajkowsky, D.M., Iwamoto, H., Ye, D., Cao, P., Schraw, W., Szabo, G., Blanke, S.R., Shao, Z. and Cover, T.L. (1999). A dominant negative mutant of *Helicobacter pylori* vacuolating toxin (VacA) inhibits VacA-induced cell vacuolation. *J. Biol. Chem*. **274,** 37736–37742.

Wang, H.J. and Wang, W.C. (2000). Expression and binding analysis of GST-*vacA* fusions reveals that the C-terminal approximately 100-residue segment of exotoxin is crucial for binding in HeLa cells. *Biochem. Biophys. Res. Commun*. **278,** 449–454.

Wang, W.C., Wang, H.J. and Kuo, C.H. (2001). Two distinctive cell binding patterns by vacuolating toxin fused with glutathione S-transferase: one high-affinity m1-specific binding and the other lower-affinity binding for variant m forms. *Biochemistry* **40,** 11887–11896.

Warren, J.R. and Marshall, B.J. (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **1,** 1273–1275.

Whiteheart, S.W., Schraw, T. and Matveeva, E.A. (2001). N-ethylmaleimide sensitive factor (NSF) structure and function. *Int. Rev. Cytol*. **207,** 71–112.

Willhite, D.C. and Blanke, S.R. (2004). *Helicobacter pylori* vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. *Cell. Microbiol*. **6,** 143–154.

Willhite, D.C., Cover, T.L. and Blanke, S.R. (2003). Cellular vacuolation and mitochondrial cytochrome c release are independent outcomes of *Helicobacter pylori* vacuolating cytotoxin activity that are each dependent on membrane channel formation. *J. Biol. Chem*. **278,** 48204–48209.

Willhite, D.C., Ye, D. and Blanke, S.R. (2002). Fluorescence resonance energy transfer microscopy of the *Helicobacter pylori* vacuolating cytotoxin within mammalian cells. *Infect. Immun*. **70,** 3824–3832.

Williams, K.A., Glibowicka, M., Li, Z., Li, H., Khan, A.R., Chen, Y.M., Wang, J., Marvin, D.A. and Deber, C.M. (1995). Packing of coat protein amphipathic and transmembrane helices in filamentous bacteriophage M13: role of small residues in protein oligomerization. *J. Mol. Biol*. **252,** 6–14.

Wirth, H.P., Beins, M.H., Yang, M., Tham, K.T. and Blaser, M.J. (1998). Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. *Infect. Immun*. **66,** 4856–4866.

Yaffe, M.B. and Smerdon, S.J. (2004). The use of *in vitro* peptide-library screens in the analysis of phosphoserine/threonine-binding domain structure and function. *Annu. Rev. Biophys. Biomol. Struct*. **33,** 225–244.

Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P.I., Wada, A. and Hirayama, T. (1997). *Helicobacter pylori* vacuolating cytotoxin binds to the 140-kDa protein in human gastric cancer cell lines, AZ-521, and AGS. Biochem. *Biophys. Res. Commun*. **238,** 629–632.

Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J. and Hirayama, T. (1999). Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase β. *J. Biol. Chem*. **274,** 36693–36699.

Yahiro, K., Wada, A., Nakayama, M., Kimura, T., Ogushi, K., Niidome, T., Aoyagi, H., Yoshino, K., Yonezawa, K., Moss, J. and Hirayama, T. (2003). Protein-tyrosine phosphatase α, RPTPα, is a *Helicobacter pylori* VacA receptor. *J. Biol. Chem*. **278,** 19183–19189.

Yahiro, K., Wada, A., Yamasaki, E., Nakayama, M., Nishi, Y., Hisatsune, J., Morinaga, N., Sap, J., Noda, M., Moss, J. and Hirayama, T. (2004). Essential domain of receptor tyrosine phosphatase β, RPTPβ, for interaction with *Helicobacter pylori* vacuolating cytotoxin. *J. Biol. Chem*. **279,** 51013–51021.

Yanagida, N., Uozumi, T. and Beppu, T. (1986). Specific excretion of *Serratia marcescens* protease through the outer membrane of *Escherichia coli. J. Bacteriol*. **166,** 937–944.

Ye, D. and Blanke, S.R. (2000). Mutational analysis of the *Helicobacter pylori* vacuolating toxin amino terminus: identification of amino acids essential for cellular vacuolation. *Infect. Immun*. **68,** 4354–4357.

Ye, D. and Blanke, S.R. (2002). Functional complementation reveals the importance of intermolecular monomer interactions for *Helicobacter pylori* VacA vacuolating activity. *Mol. Microbiol*. **43,** 1243–1253.

Ye, D., Willhite, D.C. and Blanke, S.R. (1999). Identification of the minimal intracellular vacuolating domain of the *Helicobacter pylori* vacuolating toxin. *J. Biol. Chem*. **274,** 9277–9282.

Yuan, J.P., Li, T., Li, Z.H., Yang, G.Z., Hu, B.Y., Shi, X.D., Shi, T.L., Tong, S.Q. and Guo, X.K. (2004). mRNA expression profiling reveals a role of *Helicobacter pylori* vacuolating toxin in escaping host defense. *World J. Gastroenterol*. **10,** 1528–1532.

Zheng, P.Y. and Jones, N.L. (2003). *Helicobacter pylori* strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell. Microbiol*. **5,** 25–40.

CHAPTER

# 25

# *Escherichia coli* heat-stable enterotoxin b

*J. Daniel Dubreuil*

## INTRODUCTION

Although most *Escherichia coli* strains live as symbiotic organisms in the intestine of animals including man, several pathogenic strains belonging to distinct groups are now defined on the basis of the production of specific virulence factors. Pathogenic strains belonging to different groups cause diarrhea via diverse mechanisms. Enterotoxigenic *E. coli* (ETEC) represents one of these groups. ETEC strains are important causes of traveler's diarrhea and diarrheal illnesses in children in developing countries. In addition to causing human diseases, specific ETEC strains are also responsible for severe diarrhea in domesticated and also in some wild animals (Beutin, 1999; Caprioli *et al.*, 1991; Sears and Kaper, 1996).

ETEC pathogenesis comprises two steps. First, the microorganism has to colonize the small intestine by means of specific adherence factors. Fimbrial or nonfimbrial adhesins that permit attachment to enterocytes of specific animal hosts and hence contribute to intestinal colonization are produced by ETEC. These adhesins enable ETEC to attach to receptors on the intestinal epithelium of susceptible hosts, to overcome physiological defenses such as peristaltism. Once established, ETEC strains produce one or more enterotoxins. Following interaction with specific receptors on the surface of the enterocytes, the enterotoxins will cause secretory diarrhea. Adherence of ETEC to intestinal epithelial cells is crucial for establishment of the pathogen and also for efficient delivery of toxins to their targets. Minimal histopathologic lesions are observed in the intestinal mucosa of animals infected by ETEC. In fact, microscopic observations reveal a layer of adherent bacteria covering uniformly the brush border epithelium of the small intestine with little or no visible alteration of the tissue.

ETEC cause diarrhea by production of two distinct types of enterotoxin, a heat-labile (LT) toxin with two subtypes (LTI and LTII) and a family of heat-stable (ST) enterotoxins. LT is a high molecular mass toxin (85 kDa) functionally and structurally related to *Vibrio cholerae* enterotoxin (Kaper *et al.*, 2004). STs are low-molecular mass toxins that share the property of retaining toxic activity after incubation at 100°C for 30 min, whereas the activity of LT is abrogated under the same conditions. Two types of ST, STa and STb (also known as STI and STII), based on solubility in methanol and activity in infant mice, were described. STa is soluble in methanol and active in infant mice, whereas STb is insoluble in methanol and not active in infant mice. STa has further been subdivided into STaH (or STIb) and STaP (or STIa), so named as they were isolated originally from a human or a porcine ETEC strain, respectively (see Chapter 48). These toxins are slightly different with respect to their sequence and number of amino acids. STb has no homology with STa enterotoxin at either the gene or the protein levels. This chapter will be devoted to *E. coli* heat-stable toxin b.

## STb POLYPEPTIDE

STb enterotoxins isolated from different animal species show the same nucleotide and amino acid sequences.

However, in a recent study, Fekete *et al*. (2003) isolated a swine ETEC strain that produced STb toxin where two amino acids were not conserved (His12-Asn, Lys23-Ile). The toxicity of this molecule was not determined. STb toxin has been observed in *E. coli* strains isolated principally from pigs but also from cattle (including water buffaloes), chickens, dogs, cats, ferrets, and humans (Beutin, 1999; Bradley *et al*., 2001; Dubreuil, 1997; Hammermueller *et al*., 1995).

The mature STb polypeptide comprises 48 amino acids containing four cysteine residues involved in disulfide bridge formation (Figure 25.1). The enterotoxin has a Mr of 5,200 Da. As already stated, STb bears no homology to STa enterotoxin (Dreyfus *et al*., 1992). The STb polypeptide is synthesized as a 71 amino-acid precursor comprising a 23 amino-acid signal sequence (Lee *et al*., 1983; Picken *et al*., 1983). Sukumar *et al*. (1995) indicated that the peptide spanning from Cys10 to Cys48 has full biological activity and that the first seven amino acids at the $NH_2$-terminus of the mature toxin are not involved in either the structure or toxicity of STb.

STb is a poorly immunogenic molecule, but antibodies can be produced against the mature toxin, provided numerous boosting injections are given. However, a better serological response can be obtained following immunization by either fusion proteins or proteins chemically coupled to STb (Dubreuil *et al*., 1996; Urban *et al*., 1990, 1991). In the later case, neutralizing antibodies can be obtained, but this occurs rarely when the native toxin is used for immunization. The anti-STb antibodies can neutralize STb toxicity, but are unable to neutralize STa or CT toxins (Hitotsubashi *et al*., 1992a). Yet, only one antigenic type of STb was observed.

## BIOCHEMICAL CHARACTERISTICS

For STb, the determined iso electric point of 9.6 corresponds to a highly basic protein (Handl *et al*., 1993). STb is insoluble in methanol, and the toxin loses biological activity following β-mercaptoethanol or trypsin treatment (Burgess *et al*., 1978; Dubreuil *et al*., 1991; Fujii *et al*., 1991). However, it resists acid (pH 2), alkaline (pH 12), and 8 M urea treatments (Dubreuil *et al*., 1991). STb is very susceptible to protease degradation, particularly by trypsin-like enzymes (Whipp, 1987; 1991). Out of 48 residues, the mature toxin contains 1 tyrosine, 2 phenylalanines, and no tryptophan; this results in a low absorbance at 280 nm (Handl *et al*., 1993).

### *estB* gene

The *estB* gene that encodes STb is found on heterogeneous plasmids that may also code for other properties, including other enterotoxins (i.e., LT, STa), colonization factors, drug resistance, colicin production, and transfer functions (Harnett and Gyles, 1985; Echeverria *et al*., 1985). The *estB* gene is part of a transposon of approximately 9 kb designated Tn*4521* (Lee *et al*., 1985; Hu *et al*., 1987; Hu and Lee, 1988). This transposon is flanked by defective IS*2* elements, but is nevertheless functional as the STb gene can transpose from one plasmid to another (Lee *et al*., 1985; Hu and Lee, 1988). The structural gene for STb from different clinical isolates appears to be uniform in size, but the flanking sequences are heterogeneous, suggesting that *estB* could be found on different transposons (Lee *et al*., 1985). Thus, it seems that transposition of *estB* is a mechanism by which this virulence factor is disseminated among ETEC. Spandau and Lee (1987) reported that the promoter for *estB* expression was weak. The promoter did not conform to the consensus sequence as one important base in the Pribnow box (–10 region), the final invariant T, is replaced by a G. However, the –35 region is highly homologous to the –35 consensus sequence. Thus, the STb promoter is capable of binding RNA polymerase, but seems to be a poor transcription initiator and hence very little STb is produced. Lawrence *et al*. (1990) indicated that cloning the *estB* gene into a high-expression vector downstream to the strong bacteriophage lambda $p_L$ promoter increased by 10- 20-fold the mRNA produced, but the amount of STb enterotoxin was not increased. STb synthesis by wild-type *E. coli* strains varies with the composition of the culture medium used (Busque *et al*., 1995). A repressive effect of glucose on STb production and a reversal of this effect upon addition of cAMP were noted.

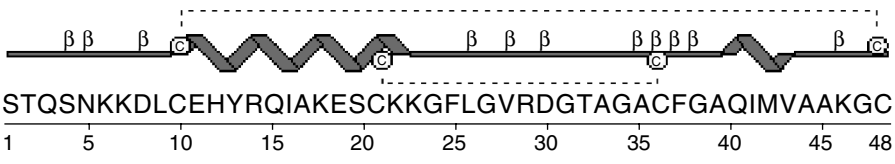



**FIGURE 25.1** Primary sequence of STb toxin showing the position of the disulfide bonds. Alpha-helices are present between Cys10-Lys22 and Gly38 and Ala44. Beta turns are also indicated.

Catabolite repression of STb was confirmed using mutant strains for adenylate cyclase and the catabolite activator protein. A DNA homology search revealed a sequence with 72% identity with the cAMP receptor protein (CRP)-binding site located 26 bp upstream of the –35 region of the transcriptional start site. The *estB* gene appears to be quite stable as laboratory strains can be studied many years (more than 15 years) without loss of the genetic trait (Dubreuil, unpublished data).

## DISULFIDE BOND FORMATION AND SECRETION OF STb

STb intramolecular disulfide bonds must be correctly formed in order to produce an active molecule. The pathway by which these bonds are formed has been established (Figure 25.2). STb polypeptide is synthesized as a 71 amino acids precursor (Lee *et al.*, 1983;

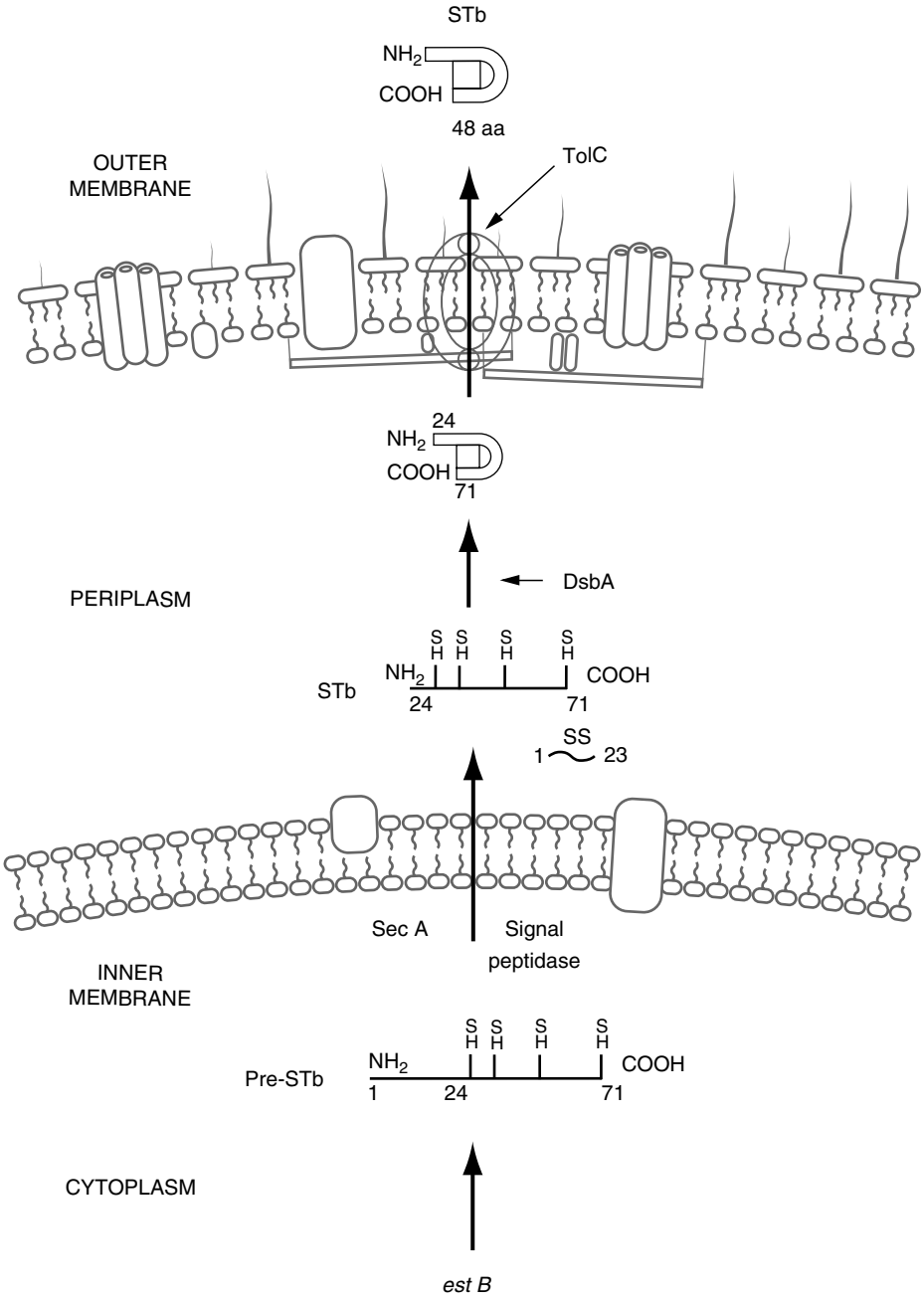



**FIGURE 25.2** Disulfide bond formation and secretory pathway for STb toxin.

Picken *et al*., 1983). The $NH_2$-terminus of pre-STb (residues 1–23) has characteristics of a signal sequence that is cleaved by a signal peptidase during export to the periplasm (Kupersztoch *et al*., 1990). Thus, an 8.1-kDa precursor (pre-STb) is converted to a transiently cell-associated 5.2-kDa form consisting of 48 amino acids. Conversion of pre-STb to cellular STb depends on the *secA* gene product. For STb, like STa, translocation of the precursor to the periplasm requires energy (Kupersztoch *et al*., 1990). These data indicate that export of STb relies on the general export pathway of *E. coli*. After STb is detected as a cell-associated molecule, an indistinguishable extracellular form becomes apparent, indicating that no proteolytic processing occurs during mobilization of STb from the periplasm to the culture supernatant. Conversion of cellular to extracellular STb does not depend on membrane potential or oxidative phosphorylation.

Foreman *et al*. (1995) obtained secretion-deficient mutants using a synthetic transposon. In *dsbA* and *tolC* defective mutants, STb was absent from the culture supernatant, indicating that these genes were required for secretion of STb. STb is effectively secreted out of the cell through the secretory system, including TolC, an outer membrane protein. Dreyfus *et al*. (1992) explored the role of the four cysteine residues in STb secretion. Cysteines were separately substituted with serine. These resulting peptides were exported to and degraded in the periplasm, suggesting that formation of disulfide bonds protected STb from protease activity. Therefore, as observed by Foreman *et al*. (1995), a *dsbA* mutant that formed disulfide bonds at a slower rate yielded a STb-negative phenotype, as the reduced form of the toxin was degraded. Similarly, Okamoto *et al*. (1995) transformed a *dsbA* mutant with a plasmid harboring *estB*. STb was not detected either in the cells or in the culture supernatant. STb production was restored by introducing the wild-type *dsbA* gene into the mutant strain, thus confirming that DsbA is involved in forming the disulfide bonds in STb and that its absence results in degradation during the secretory process. Using oligonucleotide-directed site-specific mutagenesis on the four cysteine residues, it was established by Arriaga *et al*. (1995) that the two intramolecular disulfide bonds must be formed for the efficient secretion of STb. Elimination of either one of the bonds renders the toxin susceptible to periplasmic proteolysis. Circular dichroism studies have also indicated that the integrity of the disulfide bonds is crucial for the structure and function of the toxin, as reduced STb adopted a random-coiled conformation (Sukumar *et al*., 1995). Mature STb is not associated with the cellular fraction, but found preferentially in the culture supernatant (Kupersztoch *et al*., 1990). Okamoto *et al*. (2001) studied the interaction of STb with TolC. Using deletion mutants, they analyzed the efficiency of secretion of these altered molecules into the culture supernatant. Their observations indicated that the central region of STb from amino acid 19 to 36 is involved in the secretory process, corroborating previous results showing that DsbA is necessary for STb to adopt a structure that can then cross the outer membrane.

## STb RECEPTOR

The presence and nature of the STb receptor on different tissues of various animals was investigated soon after the toxin was identified. For example, a study by Weikel *et al*. (1986b) examining the response of human adult ileal mucosa to STb in a Ussing chamber, in contrast to the piglet jejunum, which responded electrogenically to STb, indicated that human tissue showed no response. Based on these observations, the authors inferred that the adult human ileum could lack the receptor for STb. Dreyfus *et al*. (1993) suggested that a STb receptor was present on cell types of both intestinal and non-intestinal origin, as Madin-Darby canine kidney, $HT29/C_1$ human intestinal epithelial cells and primary rat pituitary cells all responded to STb. They showed that human intestinal epithelial cells did possess a receptor for STb, as a dose-dependent calcium increase was observed due to the action of STb. Hitotsubashi *et al*. (1994) identified a 25 kDa protein from the membranes of mouse intestine to which STb bound. The specificity of the interaction was corroborated by competition experiments between radiolabeled ($^{125}$I) and unlabeled toxin. Preliminary characterization of the molecule suggested that it was not a glycoprotein. The study also indicated tissue specificity, as the STb-binding protein was not found in mouse tissues such as liver, lung, spleen, and kidney. More recently, Chao and Dreyfus (1997) studied the interaction of STb with cultured human intestinal cell lines. $^{125}$I-STb bound specifically to T84 and HT-29 cells with low affinity ($\leq 10^5 M^{-1}$) to a high number of binding sites ($>10^6$ per cell), reaching equilibrium within 5–10 min at either 4, 22, or 37°C. Approximately half of the bound molecules were stably associated with the plasma membrane and/or internalized into the cytoplasm because an acidic saline treatment could not remove them. Binding and subsequent internalization were not affected by treatment of cells with trypsin, endoglycosidase F/peptide N-glycosidase F, *Vibrio cholerae* neuraminidase, tunicamycin, or sodium chlorate, indicating that protein, glycoprotein, or sulfated proteoglycans were not involved in the process. It was also shown that STb internalization was independent

of temperature, cytoskeleton rearrangement, energy, or hypertonic conditions. Overall, their results indicated that STb was probably binding to membrane lipids of the plasma membrane. The same conclusion was obtained when rat intestinal epithelial cells were studied (Chao and Dreyfus, 1999). Nevertheless, STb appeared to interact selectively with plasma membranes since STb was binding with lower affinity to CHO cells and fibroblasts than to intestinal epithelial cells. From their study, Chao and Dreyfus (1997) inferred that STb becomes stably associated with the lipid bilayer and possibly penetrates the bilayer or disrupts it sufficiently to activate directly a protein $G_{i3}$ that seems to be implicated in the mode of action of STb (Dreyfus *et al*., 1993). This hypothesis is based on the fact that the amphipathic $NH_2$-terminal helix of STb possesses a strong membrane-association potential (Segrest *et al*., 1990; Sukumar *et al*., 1995). In addition, the study of Chao and Dreyfus (1997) shed new light on the 25-kDa protein previously identified by Hitotsubashi *et al*. (1994) as the STb receptor. Based on their experimental data, the 25-kDa protein represented not a surface-specific receptor for the enterotoxin, but rather a cytoplasmic protein or a protein associated with the inner leaflet of the plasma membrane to which STb binds.

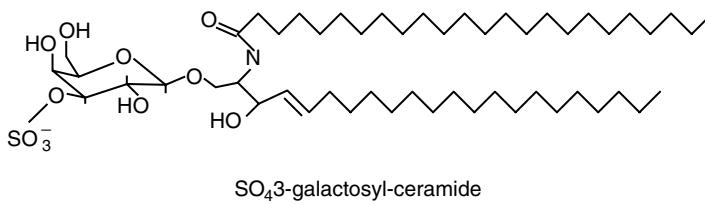
Rousset *et al*. (1998a) developed a semi-quantitative binding assay based on indirect fluorescence microscopy using biotinylated biologically active STb. The binding characteristics and the chemical nature of the molecule responsible for STb binding to the pig jejunum, the natural host tissue, were determined. The cellular complexity of sections of the pig intestinal tissue, although presenting some experimental limitations, was taken into consideration since it could lead to significantly different results from those of *in vitro* cultured intestinal epithelial cells, especially if they originated from other animal species. Using frozen sections of pig jejunum, it was demonstrated that biotinylated STb attached to microvilli. As in the study of Chao and Dreyfus (1997), binding was rapid, reaching saturation in 10 min, and the process was temperature-independent. However, binding was pH-dependent, being optimum at a pH of 5.8, which is comparable with the situation found *in vivo* for the small intestine. All pig tissues tested (including duodenum, ileum, caecum, colon, liver, lung, spleen, and kidney) showed binding toward STb, suggesting that a molecule common to these tissues could be acting as the STb receptor.

The chemical nature of the molecule involved in STb binding was studied using chemical and enzymatic treatments of the jejunum sections. Together, the data suggested that the molecule seems to be composed of a ceramide moiety comprising a terminal neuraminic acid and/or alpha-linked terminal glucose residue(s). Thus, a glycosphingolipid present in high number in the plasma membrane could specifically be recognized by STb. A high number of receptor was inferred from the fact that a 100-molar fold excess of STb could not effectively compete for binding with biotinylated STb. Chao and Dreyfus (1997) had also indicated that competition was limited, reaching approximately 50% in the presence of either 5-, 100-, or 1000-fold excess of STb. These two studies suggested that a molecule of lipidic nature present in high number in plasma membrane could represent the STb receptor.

Pursuing forward the investigation, Rousset *et al*. (1998b) evaluated the binding of STb to commercially available glycosphingolipids using a microplate-binding assay. STb binding varied greatly, depending on the molecule tested. Sulfatide ($SO_4$3-galactosyl-ceramide) was the molecule to which STb bound with greatest affinity (Figure 25.3). The reaction was dose-dependent and saturable. $GM_3$ (NeuAcα2-3GalβGlcβ1Cer) also did bind STb at approximately 55% of the level observed with sulfatide. Total lipid extraction of pig jejunum epithelium and thin-layer chromatographic analysis revealed that sulfatide is present at the surface of this tissue, as the lipid band to which STb bound was revealed with either an anti-sulfatide monoclonal antibody recognizing the $SO_4$3-galactose epitope or laminin, which specifically binds sulfated glycolipids. It thus appears that STb could bind to the pig jejunum through interaction with sulfatide. The functionality of sulfatide was also shown in the rat ligated loop assay (Rousset *et al*., 1998b). A comparative and quantitative binding assay of STb to glycolipids in microplate confirmed STb-sulfatide as a high-affinity interaction (Beausoleil and Dubreuil, 2001). As well, STb bound to $SO_4$3-lactosyl-ceramide at 76% compared to sulfatide. STb thus seems to possess a lectin-like property where molecules with terminal β-galactose are preferred for binding and the epitope recognized by STb was more specifically $SO_4$3-galactosyl. Competitive assay using $^{125}$I-labeled STb and cold toxin indicated, following a Scatchard analysis of the data, a Kd= 2-6± $1{,}5\times10^{-6}$M. This value is similar to what was determined by Chao and Dreyfus (1997) for STb binding to cells in culture ($\leq 10^{-5}$M).

To confirm the chemical structure of the STb receptor, a mass spectrometer analysis was done on the lipids islolated from pig jejunum following the method of Folch (Beausoleil *et al*., 2002b). These lipid extracts were separated on high-performance thin layer chromatographic plates, and the band migrating at the same distance as commercial sulfatide and also binding STb in overlay method were recuperated from the silica plates. The samples analyzed by mass

**FIGURE 25.3** Chemical structure of sulfatide.




spectrometry in electrospray negative-ionization mode indicated that the most abundant ions observed had *m/z* values of 779, 795, 879, and 906. Confirmation of the structure of these ions was performed using collision-induced dissociation (CID) of these ions. All the CID spectra showed a very intense 97 ion characteristic of the $HSO_4^-$ ion, along with lower intensities of the 241 ion arising from dehydration of the galactose-sulfate moiety. In comparison, to commercial bovine brain sulfatides (used as a control), ions of 795, 879, and 907 have been attributed to hydroxylated sulfatides with saturated fatty acid chain containing 16, 22, and 24 carbons, while the 779 ion corresponded to a saturated fatty acid chain of 16 carbons (Hsu *et al*., 1998). Thus, the major sulfatide ion found in pig intestine corresponding to the 795 are hydroxylated sulfatides harboring a ceramide comprising 16 carbons.

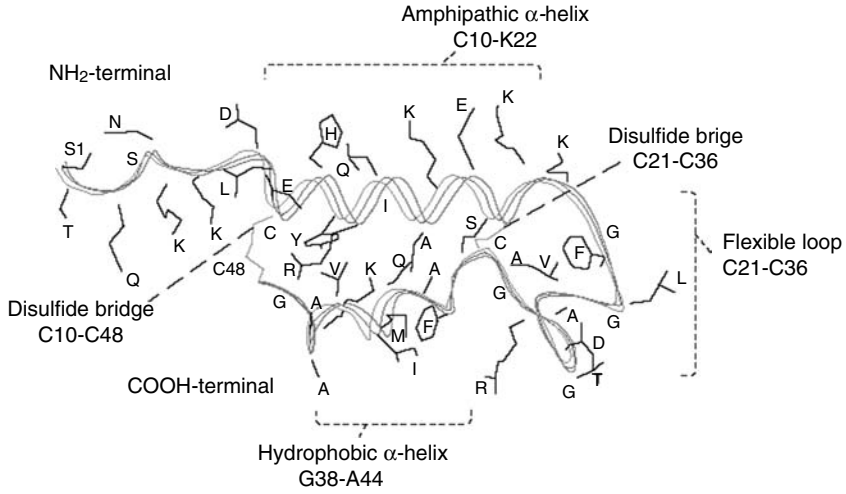
## TOXIC DOMAIN AND 3D STRUCTURE OF STb

Sukumar *et al*. (1995) determined the solution structure of STb by two- and three-dimensional NMR methods. The NMR-derived structure showed that STb is helical between residues 10 and 22 and residues 38 and 44 (Figure 25.4). The helical structure in the region 10–22 is amphipathic, exposing several polar residues to the solvent. The loop region between residues 21 and 36 contains a cluster of hydrophobic residues. Circular dichroism studies confirmed that the integrity of the disulfide bonds is crucial for the structure and function of the toxin. STb is a highly organized molecule with 73 ± 2% α-helix, 4 ± 2% β-structure and 22% remainder.

As the isoelectric point of 9.6 indicates, the side-chain of some of the basic amino-acid residues project outside the molecule. Studies have been conducted to determine the role of selected amino acids and, in particular, of the basic residues on STb toxicity. It is peculiar that, among the 48 amino acids composing mature STb, 9 are basic (one histidine, two arginines, and six lysines).

A loop defined by the disulfide bond between Cys21 and Cys36 and containing 14 amino acids (Figure 25.4), including four glycines residues and four of the nine charged residues, suggested the presence of an extended coil region based on secondary structure predictions (Dreyfus *et al*., 1992). The location of an Arg29 and Asp30 charged pair inside this loop is such that it is highly exposed in a hydrophilic environment. The authors speculated that these two

**FIGURE 25.4** NMR-derived 3D structure of STb toxin (Sukumar et al., 1995).



amino acid residues would probably be involved in receptor recognition. Using site-directed mutagenesis, amino-acid substitutions were performed on these two residues. When Arg29 was changed to serine to eliminate the charge, a significant reduction in specific activity of the mutant molecule was noted, whereas a smaller reduction of toxicity was associated with the substitution of Asp30. No alteration in the stability of the mutated molecule was noted in the intestinal loop model. Fujii *et al*. (1994) likewise investigated the role of basic amino-acid residues on STb enterotoxicity. Studies involving chemically modified STb indicated that lysine residues play an important role in STb toxicity and that the contribution of other basic residues to toxicity is relatively low. These results were confirmed using oligonucleotide-directed mutagenesis. In fact, when Lys residues at positions 18, 22, 23, and 46 were replaced by neutral amino acids, the toxicity of the molecule was reduced. Mutations of Lys22 and Lys23 resulted in a substantial alteration of the biological activity.

Based on the STb NMR structure (Sukumar *et al*., 1995), an extensive study in which amino acids were mutated in order to delineate the binding site of STb with sulfatide and the region responsible for the toxic activity was conducted (Labrie *et al*., 2001b). The focus was put on basic residues in the loop and those present in the hydrophobic α-helix that are facing the solvent. Seventeen single- or double-point mutations were produced, purified by high-pressure chromatography and analyzed by circular dichroism to assure a proper 3D structure of the molecules before testing for binding to sulfatide and toxicity. Binding to sulfatide was evaluated using a microplate binding assay, and enterotoxicity was determined, *in vivo*, using the rat loop assay (Labrie *et al*., 2001b). The results obtained indicated that hydrophobic and electrostatic interactions are important for STb attachment to sulfatide. Mutations in the hydrophobic α-helix (F37K, I41S, and M42S) decreased, as well, the binding and toxicity by more than six-fold. Basic residues in the loop defined by Cys21 and Cys36, when mutated (K22, K23, and R29), also reduced the binding and toxicity, confirming the results obtained previously by other research teams (Dreyfus *et al*., 1992; Fujii *et al*., 1994). For all STb mutants constructed and analyzed, when a reduction in binding to sulfatide was noted, a concomitant reduction in toxicity equivalent or greater was noted. Overall, this indicated that binding to sulfatide represented a prerequesite step preceding fluid secretion.

The study on delination of the binding and toxic domains of STb has established that, besides basic residues in the loop, F37, I41, and M42 are important in the expression of toxicity. As numerous toxins need to form oligomers to act on target cells, the possible implication of these hydrophobic residues in such a process for STb toxin was inferred. A study using a chemical cross-linker, [bis (sulfosuccinimidyl) suberate], and gel electrophoresis permitted the observation of protein-protein interaction of STb molecules (Labrie *et al*., 2001c). Under these conditions, STb was forming hexamers and heptamers in solution, and the process was temperature-independent. Oligomerization was observed in the absence or presence of the STb receptor, and this process was not observed in the presence of β-mercaptoethanol, indicating that STb 3D integrity is required. Interestingly, mutations in the COOH-terminal region (F37, I41, and M42) of STb, corresponding to the hydrophobic α-helix, prevented the interactions between STb molecules.

Taken together, the data suggested that the binding between STb and sulfatide is done via basic residues present in the flexible loop, while the hydrophobic α-helix is the region of the molecule involved in the STb-STb binding. The propensity of hydrophobic groups to associate due to their repulsion from the aqueous solvent could compel STb molecules to interact. This could explain the decrease in binding to sulfatide observed with polar mutants located in the hydrophobic α-helix. These mutants when tested *in vivo* showed low toxicity, indicating that oligomerization could be necessary for the expression of toxicity. These studies have demonstrated the importance of positively charged amino acids (K22, K23, and R29) in attachment to sulfatide. Thus, the initial force involved in STb-sulfatide binding could rely on electrostatic interactions. Mutating the glycines in the loop (G24, G27, G31, and G34) also decreases the attachment to sulfatide. The reduced binding could be due to the alteration of the loop flexibility for which the glycines are responsible.

## MECHANISM OF ACTION/ MEDIATORS OF SECRETION

STb is a rapidly acting toxin but of moderate action. In mouse intestinal loops, purified toxin elicits a response in 30 min and fluid accumulation reaches a maximum after about 3 h (Hitotsubashi *et al*., 1992b). Kennedy *et al*. (1984) first reported, using crude culture filtrates of STb-positive ETEC strains, that STb induced fluid secretion after 3–6 h and that the toxin did not disrupt intestinal histology. STb stimulated a cyclic nucleotide-independent secretion. Thus, STb appeared as a cytotonic toxin with properties and a mechanism of action different from that of STa. Weikel and Guerrant (1985) showed that, relative to controls, significant accumulation of $Na^+$ and $Cl^-$ occurred

intraluminally *in vivo*. Measurements of the electrolytes' content of ligated intestinal segments *in vivo* further suggested that STb stimulated bicarbonate ($HCO_3^-$) secretion. This $HCO_3^-$ secretion was also observed by other researchers (Argenzio *et al*., 1984; Weikel *et al*., 1986a). Contradictory observations were reported in different studies. For example, Whipp *et al*. (1986) observed that exposure of swine jejunum to a culture supernatant containing STb induced microscopic alterations of intestinal mucosa, in particular the loss of villous absorptive cells and partial atrophy of villi, which are consistent with the loss of absorptive capacity. The toxin was capable of causing partial villous atrophy in pigs after only 2h (Whipp *et al*., 1987; Rose *et al*., 1987).

Using light microscopy, Hitotsubashi *et al*. (1992b) observed that exposure of mouse jejunum to purify STb for 3 h caused a dilation of capillaries of the submucosa and a decrease in the thickness of the lamina propria. No cellular damage or inflammation was observed. Thus, it appears that damage to the epithelium does not occur, or rarely, with pure toxin but does when STb-containing supernatants are used (Whipp *et al*., 1987; Rose *et al*., 1987). Using the mouse intestinal loop assay model and purified toxin, Hitotsubashi *et al*. (1992b) confirmed that STb did not alter cGMP or cAMP levels in intestinal mucosal cells, indicating that the mechanism of action of STb in inducing fluid secretion differs from that of STa and of the cholera toxin (CT).

The level of prostaglandin $E_2$ ($PGE_2$) in the intestinal intraluminal fluid increased as a result of STb action, and prostaglandin synthesis inhibitors significantly reduced the response to STb. This report was the first to involve $PGE_2$ in the mechanism of action of STb. Fujii *et al*. (1995) confirmed that the quantity of $PGE_2$ produced by intestinal cells was directly related to the dose of STb administered to the mouse. In addition, the quantity of $PGE_2$ correlated with the volume of fluid released into the intestinal lumen. Levels of arachidonic acid and phosphatidic acid were also elevated by STb, indicating that arachidonic acid metabolism is stimulated by STb, possibly through phospholipase $A_2$ activity. Phosphatidic acid is also produced, from membrane phospholipids, through phospholipase C activity (Dubreuil, 1999).

Two groups have reported independently that both $PGE_2$ and 5-hydroxytryptamine (5-HT or serotonin), the latter being regarded as another secretagogue, were released into the intestinal fluid following STb administration (Harville and Dreyfus, 1995; Peterson and Whipp, 1995). Peterson and Whipp (1995) compared the secretory effects of CT, STa, and STb using the pig intestinal loop model and measured the effects of these toxins on the synthesis of cAMP, cGMP, and $PGE_2$, as well as the release of 5-HT from intestinal enterochromaffin cells. A combination of maximal doses of STa and STb yielded additive effects on fluid accumulation, suggesting different mechanisms of action. A similar additive effect on fluid accumulation and luminal release of 5-HT was noted with a combination of CT and STb. A cAMP and cGMP response to STb could not be demonstrated in either mucosal tissue or luminal fluid. Treatment of rats with ketanserin, a 5-HT receptor antagonist, reduced intestinal secretion induced by STb (Harville and Dreyfus, 1995; 1996). Nonetheless, the mode of action of STb may be somewhat similar to that of CT since the latter toxin stimulates the release of both $PGE_2$ and 5-HT, suggesting a potential effect on the enteric nervous system. Interestingly, a study by Eklund *et al*. (1985) using an *E. coli* strain producing both STa and STb showed that, at least in rats and cats, these heat-stable enterotoxins evoke secretion, in part, via activation of the enteric nervous system, as drugs influencing nervous activity significantly diminished the secretory response. Furthermore, a study using isolated mouse ileum indicated that STb could also act directly on the muscle cells of the ileal serosa increasing the spontaneous motility of the intestine and resulting in contractions (Hitotsubashi *et al*., 1992b). These contractions were not induced when the toxin was applied to the mucosa. The spontaneous motility was not inhibited by atropine, indicating that it was not the result of the excitation of cholinergic nerves. In addition, papaverine, which causes relaxation of smooth muscle, had an inhibitory effect on STb, implying that STb acts directly on muscle cells.

*In vitro* experiments suggested that STb functions by opening a G-protein–linked ($G_{i3}$, a pertussis toxin-sensitive G-protein), receptor-operated calcium channel in the plasma membrane (Dreyfus *et al*., 1993). A dose-dependent increase in intracellular $Ca^{++}$ linked to extracellular $Ca^{++}$ was observed. This process could be blocked by agents that impair GTP-binding regulatory function. Elevated intracellular $Ca^{++}$ activates phospholipases and result in the release of arachidonic acid from membrane lipids. It thus appears that the initial action of STb in the induction of diarrhea is the uptake of $Ca^{++}$ into cells. Subsequently, synthesis of $PGE_2$ and other secretagogues is stimulated, leading to diarrhea.

A study by Fujii *et al*. (1997) suggested the involvement of a $Ca^{++}$-calmodulin–dependent protein kinase II (CaMKII) in the intestinal secretory action of STb. An increase in CaMKII activity in mouse intestinal cells was noted after addition of STb in intestinal loops. A calmodulin antagonist and an inhibitor of CaMKII both reduced the observed activity of STb. Worrell and Frizzell (1991) have previously shown that $Cl^-$ secre-

tion by $Ca^{++}$ is mediated by CaMKII in cultured colonic cell lines (T84). Thus, it was proposed that after an influx of $Ca^{++}$ in the cells and activation of CaMKII through the calmodulin pathway, there would be stimulation and opening of a $Cl^-$ channel followed by subsequent fluid secretion.

At this time, which $Cl^-$ channel is stimulated by CaMKII remains unresolved. The $Ca^{++}$ pathway in which a receptor-generated increase in intracellular $Ca^{++}$ activates multifunctional CaMKII leading to phosphorylation and opening of a $Ca^{++}$-dependent $Cl^-$ channel could be implicated (Wagner *et al*., 1991, 1992; Worrell and Frizzell, 1991). These $Cl^-$ channels are entirely independent of CFTR involved in the STa mechanism of action (Wagner *et al*., 1991). Interestingly, Seidler *et al*. (1997) reported that CFTR is also involved in electrogenic $HCO_3^-$ secretion in the small intestine of mice. Three intracellular signal transduction pathways, including cAMP, cGMP, and $Ca^{++}$, were shown to stimulate $HCO_3^-$ secretion. Since $HCO_3^-$ secretion was reported as a result of STb action, it is tempting to relate the $Ca^{++}$ influx as the first step in STb action leading to $HCO_3^-$ secretion.

## PORE FORMATION AND INTERNALIZATION OF STb

Recently, an *in vitro* test was developed to evaluate the toxicity of STb as it could represent an improvement over the time-consuming and expensive *in vivo* test using rats. Thus, a model for the evaluation of toxicity using the vital stain trypan blue and cells in culture was developed (Beausoleil *et al*., 2002a). Five cell lines (including A-549, human lung; CHO, Chinese hamster ovary; IEC-18, rat ileum; HT-29, human colon; and PK-1, pig kidney) were compared for their uptake of trypan blue when STb toxin was added. Of the tested cell lines, CHO, IEC-18, and HT-29 allowed entry of the vital stain. CHO and IEC-18 were the most sensitive cells tested. Stain uptake was dose-dependent with a maximal effect at 4 μg of STb for $10^6$ cells. Maximal stain uptake was observed after 2 h. Pre-treatment of CHO cells with sulfatase (removing the sulfate groups of cell surface-exposed molecules including sulfatide) completely inhibited trypan blue intake in presence of STb. Thus, it was concluded that sulfatide, the functional receptor for STb, must be present and not altered for the entry of the trypan blue into the CHO cells. When STb mutants (17 single- or double-point mutations) were tested for relative toxicity using trypan blue exclusion assay and the rat loop assay on CHO cells, a good correlation was observed between the two bioassays. In fact, the biological activity observed in the rat model was correlated to the trypan blue uptake assay in CHO cells ($R^2$=0.78) for STb toxin and mutants tested. The variance analysis statistical test indicated a significant correlation between the two bioassays ($Fc \geq F_{0.005}$). These results suggested that the trypan blue uptake bioassay could be used to evaluate the biological activity of STb toxin and mutants. Using a MTT viability test based on the activity of mitochondrial succinate-tetrazolium reductase system, it was clear that the metabolic activity of STb-treated cells was not significally altered, as the MTT assay did not indicate any cell mortality.

An electrophysiological study using planar lipid bilayers was conducted in order to elucidate the mechanism of entry of trypan blue inside STb-treated cells (Labrie *et al*., 2001a). The receptor for STb was reconstituted into large unilamellar vesicles made of PE, which were osmotically fused to PE:PC:cholesterol (7:2:1). Upon addition of STb, on both side of the membrane, well-resolved channel currents were observed when voltage was applied. The data indicated that under 1 KCl symmetrical conditions, the STb main conductance was 180 pS. STb channel activity appeared to be voltage-dependent, being mostly open at all voltages with a tendency to close for longer periods of time at higher negative voltages. Thus, STb may permeabilize eukaryotic cell membrane by disruption of the lipid bilayer, as was also suggested by Chao and Dreyfus (1999). The observation that STb forms oligomers (Labrie *et al*., 2001c) can indicate that STb may allow formation of pores that can alter the cellular membrane, permitting trypan blue intake.

The fate of STb injected in rat intestinal loops was followed using anti-STb gold labeled antibodies and transmission electron microscopy (Labrie *et al*., 2002). STb toxin was injected into rat intestinal loops and after 3h the animal was euthanized. The tissue was fixed and cut using an ultramicrotome. Toxin detection was done with anti-STb antibodies labeled with colloidal gold. Internalization of STb toxin and also of MBP-STb fusion protein was observed. Negative controls (without toxin or without antibodies) gave negative results. A mutant, I41E-M42R, with reduced hydrophobicity and unable to form oligomers but with residual toxicity (approximately 11% compared to STb) was not internalized. These results could mean that oligomerization of STb precedes and is required for internalization. No precise subcellular compartment could be determined as the final destination of the STb toxin.

## CONCLUSION

The heat-stable enterotoxin STb produced by ETEC *E. coli* induces intestinal fluid accumulation, resulting

in diarrheal illnesses and diseases in both humans and animals. From the recently acquired knowledge on STb toxin and on its receptor, we can propose a mechanistic view of the interaction of the toxin with the eukaryotic cell (Figure 25.5). Thus, STb toxin would form hexamers or heptamers in solution, the aqueous milieu compelling the interaction of hydrophobic α-helices together. These oligomers could be formed even in the absence of the receptor. Then oligomers would bind to sulfatide present on the cell surface. This interaction would involve basic residues (K22, K23, and R29) present in the loop (between C21–C36) and the galactose 3-sulfate moiety through initial electrostatic interactions. Then the STb oligomer or monomer would be inserted in the lipidic membrane bilayer. This process could destabilize momentarily the membrane, or pores could be produced, explaining the trypan blue uptake observed in cell in culture. Internalization would then be completed, and STb toxin would be found inside the eukaryotic cell as was observed by transmission electron microscopy (Labrie *et al.*, 2002). On the other hand, as in PLB, current channels were observed, pores could

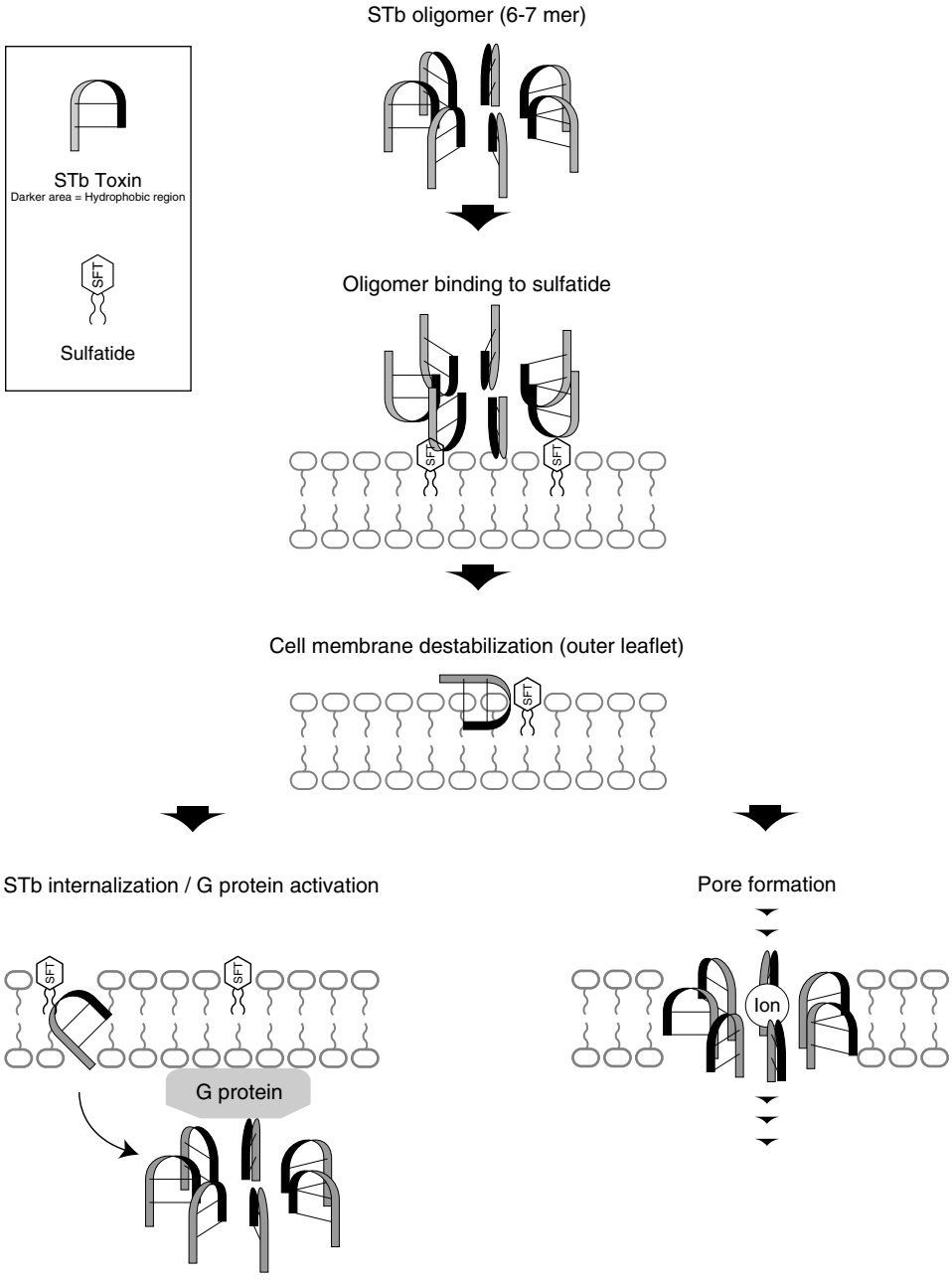



**FIGURE 25.5** Schematic illustration of the proposed mechanistic view of the interaction between STb and the eukaryotic cell based on racently acquired knowledge.

also be formed and selectively allow the passage of ions as $Ca^{++}$. Internalized toxin would now have a direct access to cytoplasmic components of the eukaryotic cell. For example, a direct access of STb to the already implied $G_{i3}$ protein would now be possible.

## ACKNOWLEDGMENTS

The author would like to acknowledge the contribution of graduate students and collaborators to the work presented in this review. In particular, É. Rousset, H.-É. Beausoleil, V. Labrie, J. Harel, F. Lépine, and J.-L. Schwartz. P. Sasseville realized the graphic work. The work has been supported by grants from the Natural Sciences and Engineering Research Council of Canada.

## REFERENCES

Argenzio, R.A., Liacos, J., Berschneider, H.M., Whipp, S.C. and Robertson, D.C. (1984). Effect of heat-stable enterotoxin of *Escherichia coli* and theophylline on ion transport in porcine small intestine. *Can. J. Comp. Med*. **48,** 14–22.

Arriaga, Y.L., Harville, B.A. and Dreyfus, L.A. (1995). Contribution of individual disulfide bonds to biological action of *Escherichia coli* heat-stable enterotoxin B. Infect. *Immun*. **63,** 4715–4720.

Beausoleil, H.E. and Dubreuil, J.D. (2001). *In vitro* binding characteristics and affinity for sulfatide of *Escherichia coli* STb enterotoxin. *Receptors Channels* **7,** 401–411.

Beausoleil, H.E., Labrie, V. and Dubreuil, J.D. (2002a). Trypan blue uptake by Chinese hamster ovary cultured epithelial cells: a cellular model to study *Escherichia coli* STb enterotoxin. *Toxicon* **40,** 185–191.

Beausoleil, H.-E., Lépine, F. and Dubreuil, J.D. (2002b). LC-MS analysis of pig intestine sulfatides: Interaction with *Escherichia coli* STb enterotoxin and characterization of molecular species present. *FEMS Microbiol. Lett*. **209,** 183–188.

Beutin, L. (1999). *Escherichia coli* as a pathogen in dogs and cats. *Vet. Res*. **30,** 285–298.

Bradley, G.A., Orr, K., Reggiardo, C. and Glock, R.D. (2001). Enterotoxigenic *Escherichia coli* infection in captive black-footed ferrets. *J. Wildl. Dis*. **37,** 617–620.

Burgess, M.N., Bywater, R.J., Cowley, C.M., Mullan, N.A. and Newsome, P.M. (1978). Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits, and calves. *Infect. Immun*. **21,** 526–531.

Busque, P., Letellier, A., Harel, J. and Dubreuil, J.D. (1995). Production of *Escherichia coli* STb enterotoxin is subject to catabolite repression. *Microbiology* **141,** 1621–1627.

Caprioli, A., Donelli, G., Falbo, V., Passi, C., Pagano, A. and Mantovani, A. (1991). Antimicrobial resistance and production of toxins in *Escherichia coli* strains from wild ruminants and the alpine marmot. *J. Wildl. Dis*. **27,** 324–327.

Chao, K.L. and Dreyfus, L.A. (1997). Interaction of *Escherichia coli* heat-stable enterotoxin B with cultured human intestinal epithelial cells. *Infect. Immun*. **65,** 3209–3217.

Chao, K.L. and Dreyfus, L.A. (1999). Interaction of *Escherichia coli* heat-stable enterotoxin B with rat intestinal epithelial cells and membrane lipids. *FEMS Microbiol. Lett*. **172,** 91–97.

Dreyfus, L.A., Urban, R.G., Whipp, S.C., Slaughter, C., Tachias, K. and Kupersztoch, Y.M. (1992). Purification of the STB enterotoxin of *Escherichia coli* and the role of selected amino-acids on its secretion, stability, and toxicity. *Mol. Microbiol*. **6,** 2397–2406.

Dreyfus, L.A., Harville, B., Howard, D.E., Shaban, R., Beatty, D.M. and Morris, S.J. (1993). Calcium influx mediated by the *Escherichia coli* heat-stable enterotoxin B (STB). *Proc. Natl. Acad. Sci. USA* **90,** 3202–3206.

Dubreuil, J.D. (1997). *Escherichia coli* STb enterotoxin. *Microbiology* **143,** 1783–1795.

Dubreuil, J.D. (1999). *Escherichia coli* STb toxin and prostaglandin production. *Microbiology* **145,** 1507–1508.

Dubreuil, J.D., Fairbrother, J.M., Lallier, R. and Larivière, S. (1991). Production and purification of heat-stable enterotoxin b from a porcine *Escherichia coli* strain. *Infect. Immun*. **59,** 198–203.

Dubreuil, J.D., Letellier, A. and Harel, J. (1996). A recombinant *Escherichia coli* heat-stable enterotoxin b (STb) fusion protein eliciting neutralizing antibodies. *FEMS Immunol. Med. Microbiol*. **13,** 317–323.

Echeverria, P., Seriwatana, J., Taylor, D.N., Tirapat, C. and Rowe, B. (1985). *Escherichia coli* contains plasmids coding for heat-stable b, other enterotoxins, and antibiotic resistance. *Infect. Immun*. **48,** 843–846.

Eklund, S., Jodal, M. and Lundgren, O. (1985). The enteric nervous system participates in the secretory response to the heat stable enterotoxins of *Escherichia coli* in rats and cats. *Neuroscience* **14,** 673–681.

Fekete, P.Z., Schneider, G., Olasz, F., Blum-Oehler, G., Hacker, J.H. and Nagy, B. (2003). Detection of a plasmid-encoded pathogenicity island in F18+ enterotoxigenic and verotoxigenic *Escherichia coli* from weaned pigs. *Int. J. Med. Microbiol*. **293,** 287–298.

Foreman, D.T., Martinez, Y., Coombs, G., Torres, A. and Kupersztoch, Y.M. (1995). TolC and DsbA are needed for the secretion of STB, a heat-stable enterotoxin of *Escherichia coli. Mol. Microbiol*. **18,** 237–245.

Fujii, Y., Hayashi, M., Hitotsubashi, S., Fuke, Y., Yamanaka, H. and Okamoto, K. (1991). Purification and characterization of *Escherichia coli* heat-stable enterotoxin II. *J. Bacteriol*. **173,** 5516–5522.

Fujii, Y., Kondo, Y. and Okamoto, K. (1995). Involvement of prostaglandin E2 synthesis in the intestinal secretory action of *Escherichia coli* heat-stable enterotoxin II. *FEMS Microbiol. Lett*. **130,** 259–265.

Fujii, Y., Nomura, T., Yamanaka, H. and Okamoto, K. (1997). Involvement of Ca(2+)-calmodulin-dependent protein kinase II in the intestinal secretory action of *Escherichia coli* heat-stable enterotoxin II. *Microbiol. Immunol*. **41,** 633–636.

Fujii, Y., Okamuro, Y., Hitotsubashi, S., Saito, A., Akashi, N. and Okamoto, K. (1994). Effect of alterations of basic amino acid residues of *Escherichia coli* heat-stable enterotoxin II on enterotoxicity. *Infect. Immun*. **62,** 2295–2301.

Hammermueller, J., Kruth, S., Prescott, J. and Gyles, C. (1995). Detection of toxin genes in *Escherichia coli* isolated from normal dogs and dogs with diarrhea. *Can. J. Vet. Res*. **59,** 265–270.

Handl, C.E., Harel, J., Flock, J.I. and Dubreuil, J.D. (1993). High yield of active STb enterotoxin from a fusion protein (MBP-STb) expressed in *Escherichia coli. Protein Expr. Purif*. **4,** 275–281.

Harnett, N.M. and Gyles, C.L. (1985). Enterotoxin plasmids in bovine and porcine enterotoxigenic *Escherichia coli* of O groups 9, 20, 64, and 101. *Can. J. Comp. Med*. **49,** 79–87.

Harville, B.A. and Dreyfus, L.A. (1995). Involvement of 5-hydroxytryptamine and prostaglandin E2 in the intestinal secretory action of *Escherichia coli* heat-stable enterotoxin B. *Infect. Immun*. **63,** 745–750.

Harville, B.A. and Dreyfus, L.A. (1996). Release of serotonin from RBL-2H3 cells by the *Escherichia coli* peptide toxin STb. *Peptides* **17,** 363–366.

Hitotsubashi, S., Akagi, M., Saitou, A., Yamanaka, H., Fujii, Y. and Okamoto, K. (1992a). Action of *Escherichia coli* heat-stable enterotoxin II on isolated sections of mouse ileum. *FEMS Microbiol. Lett*. **69,** 249–252.

Hitotsubashi, S., Fujii, Y. and Okamoto, K. (1994). Binding protein for *Escherichia coli* heat-stable enterotoxin II in mouse intestinal membrane. *FEMS Microbiol. Lett*. **122,** 297–302.

Hitotsubashi, S., Fujii, Y., Yamanaka, H. and Okamoto, K. (1992b). Some properties of purified *Escherichia coli* heat-stable enterotoxin II. *Infect. Immun*. **60,** 4468–4474.

Hsu, F.F., Bohrer, A. and Turk, J. (1998). Electrospray ionization tandem mass spectrometric analysis of sulfatide. Determination of fragmentation patterns and characterization of molecular species expressed in brain and in pancreatic islets. *Biochim. Biophys. Acta* **1392,** 202–216.

Hu, S.T. and Lee, C.H. (1988). Characterization of the transposon carrying the STII gene of enterotoxigenic *Escherichia coli*. *Mol. Gen. Genet*. **214,** 490–495.

Hu, S.T., Yang, M.K., Spandau, D.F., and Lee, C.H. (1987). Characterization of the terminal sequences flanking the transposon that carries the *Escherichia coli* enterotoxin STII gene. *Gene* **55,** 157–167.

Kaper, J.B., Nataro, J.P. and Mobley, H.L. (2004). Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol*. **2,** 123–140.

Kennedy, D.J., Greenberg, R.N., Dunn, J.A., Abernathy, R., Ryerse, J.S. and Guerrant, R.L. (1984). Effects of *Escherichia coli* heat-stable enterotoxin STb on intestines of mice, rats, rabbits, and piglets. *Infect. Immun*. **46,** 639–643.

Kupersztoch, Y.M., Tachias, K., Moomaw, C.R., Dreyfus, L.A., Urban, R., Slaughter, C. and Whipp, S. (1990). Secretion of methanol-insoluble heat-stable enterotoxin (STB): energy- and secA dependent conversion of pre-STB to an intermediate indistinguishable from the extracellular toxin. *J. Bacteriol*. **172,** 2427–2432.

Labrie, V., Potvin, L., Harel, J., Dubreuil, J.D. and Schwartz, J.-L. (2001a). Enterotoxin b of *Escherichia coli* (STb) forms ion channels in planar lipid bilayers. *10th European Workshop Conference on Bacterial Protein Toxins*., Bohon, Belgium, A54.

Labrie, V., Beausoleil, H.E., Harel, J. and Dubreuil, J.D. (2001b). Binding to sulfatide and enterotoxicity of various *Escherichia coli* STb mutants. *Microbiology* **147,** 3141–3148.

Labrie, V., Harel, J. and Dubreuil, J.D. (2001c). Oligomerization of *Escherichia coli* enterotoxin b through its C-terminal hydrophobic alpha-helix. *Biochim. Biophys. Acta* **1535,** 128–133.

Labrie, V., Harel, J. and Dubreuil, J.D. (2002). *Escherichia coli* heat-stable enterotoxin b (STb) *in vivo* internalization within rat intestinal epithelial cells. *Vet. Res*. **33,** 223–228.

Lawrence, R.M., Huang, P.T., Glick, J., Oppenheim, J.D. and Maas, W.K. (1990). Expression of the cloned gene for enterotoxin STb of *Escherichia coli*. *Infect. Immun*. **58,** 970–977.

Lee, C.H., Hu, S.T., Swiatek, P.J., Moseley, S.L., Allen, S.D. and So, M. (1985). Isolation of a novel transposon which carries the *Escherichia coli* enterotoxin STII gene. *J. Bacteriol*. **162,** 615–620.

Lee, C.H., Moseley, S.L., Moon, H.W., Whipp, S.C., Gyles, C.L. and So, M. (1983). Characterization of the gene encoding heat-stable toxin II and preliminary molecular epidemiological studies of enterotoxigenic *Escherichia coli* heat-stable toxin II producers. *Infect. Immun*. **42,** 264–268.

Okamoto, K., Baba, T., Yamanaka, H., Akashi, N. and Fujii, Y. (1995). Disulfide bond formation and secretion of *Escherichia coli* heat-stable enterotoxin II. *J. Bacteriol*. **177,** 4579–4586.

Okamoto, K., Yamanaka, H., Takeji, M. and Fuji, Y. (2001). Region of heat-stable enterotoxin II of *Escherichia coli* involved in translocation across the outer membrane. *Microbiol. Immunol*. **45,** 349–355.

Peterson, J.W. and Whipp, S.C. (1995). Comparison of the mechanisms of action of cholera toxin and the heat-stable enterotoxins of *Escherichia coli*. *Infect. Immun*. **63,** 1452–1461.

Picken, R.N., Mazaitis, A.J., Maas, W.K., Rey, M. and Heyneker, H. (1983). Nucleotide sequence of the gene for heat-stable enterotoxin II of *Escherichia coli*. *Infect. Immun*. **42,** 269–275.

Rose, R., Whipp, S.C. and Moon, H.W. (1987). Effects of *Escherichia coli* heat-stable enterotoxin b on small intestinal villi in pigs, rabbits, and lambs. *Vet. Pathol*. **24,** 71–79.

Rousset, E. and Dubreuil, J.D. (1999). Evidence that *Escherichia coli* STb enterotoxin binds to lipidic components extracted from the pig jejunal mucosa. *Toxicon* **37,** 1529–1537.

Rousset, E., Harel, J. and Dubreuil, J.D. (1998a). Sulfatide from the pig jejunum brush border epithelial cell surface is involved in binding of *Escherichia coli* enterotoxin b. *Infect. Immun*. **66,** 5650–5658.

Rousset, E., Harel, J. and Dubreuil, J.D. (1998b). Binding characteristics of *Escherichia coli* enterotoxin b (STb) to the pig jejunum and partial characterization of the molecule involved. *Microb. Pathog*. **24,** 277–288.

Sears, C.L. and Kaper, J.B. (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol. Rev*. **60,** 167–215.

Segrest, J.P., De Loof, H., Dohlman, J.G., Brouillette, C.G. and Anantharamaiah, G.M. (1990). Amphipathic helix motif: classes and properties. *Proteins* **8,** 103–117.

Seidler, U., Blumenstein, I., Kretz, A., Viellard–Baron, D., Rossmann, H., Colledge, W.H., Evans, M., Ratcliff, R. and Gregor, M. (1997). A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca(2+)-dependent $HCO3^-$ secretion. *J. Physiol*. **505,** 411–423.

Spandau, D.F. and Lee, C.H. (1987). Determination of the promoter strength of the gene encoding *Escherichia coli* heat-stable enterotoxin II. *J. Bacteriol*. **169,** 1740–1744.

Sukumar, M., Rizo, J., Wall, M., Dreyfus, L.A., Kupersztoch, Y.M. and Gierasch, L.M. (1995). The structure of *Escherichia coli* heat-stable enterotoxin b by nuclear magnetic resonance and circular dichroism. *Protein Sci*. **4,** 1718–1729.

Urban, R.G., Dreyfus, L.A. and Whipp, S.C. (1990). Construction of a bifunctional *Escherichia coli* heat-stable enterotoxin (STb)-alkaline phosphatase fusion protein. *Infect. Immun*. **58,** 3645–3652.

Urban, R.G., Pipper, E.M. and Dreyfus, L.A. (1991). Monoclonal antibodies specific for the *Escherichia coli* heat-stable enterotoxin STb. *J. Clin. Microbiol*. **29,** 1963–1968.

Wagner, J.A., Cozens, A.L., Schulman, H., Gruenert, D.C., Stryer, L. and Gardner, P. (1991). Activation of chloride channels in normal and cystic fibrosis airway epithelial cells by multifunctional calcium/calmodulin-dependent protein kinase. *Nature* **349,** 793–796.

Wagner, J.A., McDonald, T.V., Nghiem, P.T., Lowe, A.W., Schulman, H., Gruenert, D.C., Stryer, L. and Gardner, P. (1992). Antisense oligodeoxynucleotides to the cystic fibrosis transmembrane conductance regulator inhibit cAMP-activated but not calcium-activated chloride currents. *Proc. Natl. Acad. Sci. USA* **89,** 6785–6789.

Weikel, C.S. and Guerrant, R.L. (1985). STb enterotoxin of *Escherichia coli*: cyclic nucleotide-independent secretion. *Ciba Found. Symp*. **112,** 94–115.

Weikel, C.S., Nellans, H.N. and Guerrant, R.L. (1986a). *In vivo* and *in vitro* effects of a novel enterotoxin, STb, produced by *Escherichia coli*. *J. Infect. Dis*. **153,** 893–901.

Weikel, C.S., Tiemens, K.M., Moseley, S.L., Huq, I.M. and Guerrant, R.L. (1986b). Species specificity and lack of production of

STb enterotoxin by *Escherichia coli* strains isolated from humans with diarrheal illness. *Infect. Immun*. **52,** 323–325.

Whipp, S.C., Moseley, S.L. and Moon, H.W. (1986). Alterations in jejunal epithelium of three-week-old pigs induced by pig-specific, mouse-negative, heat-stable *Escherichia coli* enterotoxin. *Am. J. Vet. Res*. **47,** 615–618.

Whipp, S.C. (1987). Protease degradation of *Escherichia coli* heat-stable, mouse-negative, pig-positive enterotoxin. *Infect. Immun*. **55,** 2057–2060.

Whipp, S.C. (1991). Intestinal responses to enterotoxigenic *Escherichia coli* heat-stable toxin b in non-porcine species. *Am. J. Vet. Res*. **52,** 734–737.

Worrell, R.T. and Frizzell, R.A. (1991). CaMKII mediates stimulation of chloride conductance by calcium in T84 cells. *Am. J. Physiol*. **260,** C877–882.

SECTION III

# TOXINS ACTING ON THE SURFACE OF TARGET CELLS (EXCEPT SUPERANTIGENS)

CHAPTER

# 26

# Paradigms and classification of bacterial membrane-damaging toxins

*Joseph E. Alouf*

## INTRODUCTION

The history of membrane-damaging toxins (MDTs) spans a period of about a century (Bernheimer 1996). To our knowledge, the first membrane-damaging activity of a bacterial culture fluid on erythrocytes was characterized by Paul Ehrlich (1888) and Alexandre Marmorek (1902) in the culture fluids of *Clostridium tetani* and *Streptococcus pyogenes*, respectively. The products involved were tetanolysin for the former and streptolysin S or O (or both) for the latter (see Chapters 36 and 42 in this volume). In this introductory chapter, some prominent features of MDTs are briefly outlined. A detailed description of these toxins is presented in (Chapters 27 to 47) of this volume.

## HISTORICAL BACKGROUND

MDTs were first identified by virtue of their lytic action *in vitro* on erythrocyte suspensions (in saline) of human or other animal species and the appearance of zones of hemolysis around bacterial colonies growing on blood agar plates (Alouf, 1977). This approach is still used for phenotypic characterization of hemolytic bacteria and consequently for the identification of relevant hemolytic toxins. However, it became clear from an early stage that many if not all bacterial hemolysins acted on cells other than erythrocytes. Many of them also caused tissue damage or death when injected into experimental animals, thereby justifying their status as "toxins."

The term "hemolysin," generally used in the literature until the 1970s (and sometimes even to date), appeared somewhat restrictive and inappropriate as a general term for MDTs. Certain members of this group are inactive on erythrocytes, while they damage other cells, such as leucocytes. This led to coining the term "leucocidin" for some of them, e.g., *S. aureus* leucocidins, *Pseudomonas aeruginosa* leucocidin also named cytotoxin, and the RTX leucotoxins of various Gram-negative bacterial species (Prevost *et al*. 2001; Xiong *et al*., 1994; Thumbikat *et al*. 2003). Therefore, Alan Bernheimer, a pioneer in the field of membrane-damaging toxins, proposed the terms "cytolysin" or "cytolytic toxin" in an attempt to describe more accurately the biological activity of MDTs (Bernheimer, 1970).

A new period in the study of bacterial cytolysins started at the turn of the 1970s. It concerned the mechanism of action of these proteins on various eucaryotic cells or cell lines, as well as on isolated cell membranes (ghosts) and artificial model membrane systems (phospholipid films and liposomes) investigated by a variety of biochemical, physical, and electron microscopic techniques. A considerable amount of literature is available for this important field (Alouf, 1977; Thelestam and Möllby, 1979; Alouf *et al*., 1984, 1989; Duncan, 1984; Bhakadi and Tranum-Jensen, 1988, 1991; Harshman *et al*., 1989; Sekiya *et al*., 1993, 1996; Walker

and Bayley, 1995; Morgan *et al*., 1994; Dobereiner *et al*., 1996; Palmer *et al*., 1996, 1998; Valeva *et al*., 1997; Vandana *et al*., 1997; Nakamura *et al*., 1998; Staali *et al*., 1998; Shepard *et al*., 1998; Gilbert *et al*., 1998; Rossjohn *et al*., 1998, 1999; Oscarsson, 1999; Jacobs *et al*., 1999; Alouf and Palmer, 1999; Menestrina, 2000; Billington *et al*., 2000; van der Goot, 2000; Hertle, 2000, 2002; Manteca *et al*., 2002; Heuck, 2003; Czajkowsky *et al*., 2004).

## GENERAL FEATURES OF THE MEMBRANE–DAMAGING TOXINS

MDTs are produced by a great number of both Gram-positive and Gram-negative bacterial species. To date, this superfamily of toxins comprises 122 toxins (about 36%) versus the repertoire of 339 bacterial protein toxins so far identified. Thus, the superfamily of MDTs constitutes the most important funtional group of bacterial protein toxins.

MDTs are characterized by their property to damage or disrupt the integrity of the cytoplasmic membrane of various eucaryotic cells (impairment of osmotic balance), reflected by cell swelling and subsequently cell lysis and the release of intracytoplasmic content, particularly hemoglobin in the case of erythrocytes and various macromolecules such as lactate dehydrogenases from various cells during the lytic process (Launay *et al*., 1984; Bhakdi and Tranum-Jensen, 1988; Menestrina and Vécsey-Semjén, 1999; van der Goot, 2003; Alouf, 1980, 2001).

Moreover, following the damage of the cytoplasmic membranes of target cells, many MDTs are able to disrupt the membranes of intracytoplasmic organelles, such as mitochondria, lysosomes, phagosomes, dense bodies, etc., leading to the release of a considerable array of pharmacologically active substances in the surrounding medium of the cells (enzymes, inflammatory factors, cytokines, serotonin) (Alouf, 1980; Launay and Alouf, 1979; Bhakdi *et al*., 1989).

### Toxin release and molecular features

The majority of PFTs are simple single-chain proteins secreted by the bacteria (extracellular toxins), but some of them may remain associated at the suface of these microorganisms. This is the case of the cell-bound form of streptolysin S (Calandra and Cole, 1981; and Chapter 42 of this volume), *Bordetella pertussis* adenylate cyclase-hemolysin (Locht, 1999). Other cytolysins remain strictly located in the cytoplasm, e.g., pneumolysin from *Streptococcus pneumoniae* (Johnson, 1977). This toxin is released in the surrounding medium after cell autolysis. Interestingly, *E. coli* ClyA cytotoxin is surface exposed and exported from bacterial cells in outer membrane vesicles (Wai *et al*., 2003).

The molecular weights of PFTs range from about 2.5 kDa for *S. aureus* δ-toxin (Freer and Birkbeck, 1982; Alouf *et al*., 1989; Dufourcq *et al*., 1999) up to about 177 kDa for *B. pertussis* adenylate cyclase-hemolysin (Locht, 1999). However, many PFTs show a broad diversity of molecular topology, genomic encoding, structural architecture, and functional originality, as described in subsequent chapters.

## PATHOGENIC EFFECTS OF MEMBRANE-DAMAGING TOXINS

Several lines of experimental evidence and clinical observations demonstrated (particularly by genetic deletions) that many MDTs play a pivotal role in bacterial virulence and pathogenesis. In experimental animal models, many toxins elicited the damage of host's cells, tissues, and organs, provoked apoptosis, organelle vacuolation, as well as the production of inflammatory mediators, including various cytokines. Animal death was also recorded (Bhakdi and Tranum-Jensen, 1991; Coote, 1996; Paton, 1996; Rubins *et al*., 1996; Schmiel and Miller, 1999; Ellemor *et al*., 1999; Jacobs *et al*., 1999; van der Goot, 2003; Oxhamre and Richter-Dahlfors, 2003).

### Lethal effects of MDTs

The lethality in humans of various MDTs is well established as dramatically illustrated by the tragedy (1928) in the small Australian town of Bundaberg in which 21 children died in the course of vaccination against diphtheria. The vaccine was contaminated with *Staphylococcus aureus*. The investigation of the cause of the fatalities established that death resulted from an overwhelming toxemia due to hemolyic factors (most probably α-toxin) at the early stage of the invasion of the organism (see Arbuthnott, 1970). Other typical examples among many others is the major involvement of *Clostridium perfringens* hemolytic α-toxin (phospholipase C) in the pathogenesis of gas gangrene (Bryant *et al*., 2000a, b; Stevens and Bryant, 2002; and Chapter 5 of this volume), *C. perfringens* β-2 toxin, and *C. septicum* α-toxin (Tweten, 2001; Manteca *et al*., 2002). RTX cytolysins from *Pasteurellaceae* and other species are also involved in animal and human diseases including lethality (Frey and Kuhnert, 2002; Thumbikat *et al*., 2003).

### Biological and pharmacological effects of sublytic concentrations of PFTs on target cells

The most important pathogenic effects of PFTs do not primarily concern the damage of host's erythocytes (hemolysis) as initially thought. They rather reflect the damage inflicted at sublytic doses to host's cells, which are very likely more representative of toxin concentrations *in vivo* (Billington *et al*., 2000; Oxhamre and Richter-Dahlfors, 2003). Many effectors are released by toxin-damaged cells, particularly immune system cells. These products among others are those of the cytokine network, nitric oxide, and certain key molecules in cell-signaling (Henderson *et al*., 1997; Kayal *et al*., 1999; Bryant *et al*., 2003). This is the case for:

1. *S. aureus* α-toxin (Bhakdi and Tranum-Jensen, 1991; Suttorp *et al*., 1993) and leucocidins (König *et al*., 1994b; Staali *et al*., 1998; Prévost *et al*., 2001)
2. Listeriolysin O (Nishibori *et al*., 1996; Tang *et al*., 1996; Weiglein *et al*., 1997; Tanabe *et al*., 1999; Sibelius *et al*., 1999; Jacobs *et al*., 1999)
3. RTX cytolysins, particularly *E. coli* α-hemolysin (Grimminger *et al*., 1991; König *et al*., 1994a; Coote, 1996; May *et al*., 1996; Lally *et al*., 1999; Oxhamre and Richter-Dahlfors, 2003)
4. *C. perfringens* α-toxin and perfringolysin O (Bryant *et al*., 2003; Rossjohn *et al*., 1999; Ellemor *et al*., 1999); *C. perfringens* β–2-toxin (Manteca *et al*., 2002)
5. Streptolysin O (Hackett and Stevens, 1992; Ruiz *et al*., 1998; Alouf and Palmer, 1999) and pneumolysin (Rubins *et al*., 1996; Alexander *et al*., 1998)
6. *Aeromonas hydrophila* aerolysin (Nelson *et al*., 1999; Buckley, 1999; Fivaz *et al*., 2001)
7. *B. cereus* cytotoxin K (Lund *et al*., 2000) and the *E. coli* cytolytic protein ClyA (Lai *et al*., 2000)

## CLASSIFICATION AND REPERTOIRE OF THE SUPERFAMILY OF MEMBRANE–DAMAGING TOXINS

The progress in the study of the mechanisms of membrane damage of target cells by this wide group of proteins allowed us to distinguish three families of MDTs (Arbuthnott, 1982; Bernheimer and Rudy, 1986; Rowe and Welch, 1994):

(i) The family of toxins exhibiting a detergent-like (surfactant) activity resulting in membrane "solubilization" and (or) partial insertion into the hydrophobic regions of target membranes. This appears to be the case for the 26-amino acid delta- and delta-like toxins of *Staphylococcus aureus, S. haemolyticus*, and *S. lugdunensis* (Freer and Arbuthnott, 1983; Alouf *et al*., 1989; Dufourcq *et al*., 1999), the heat-stable hemolysin from *Pseudomonas aeruginosa* (Rowe and Welch, 1994), and *Bacillus subtilis* cyclolipopeptides (surfactins, iturins, mycosubtilins, bacillomycins) (Bernheimer and Rudy, 1986; Sheppard *et al*., 1991; Maget-Dana and Peypoux, 1994; Menestrina and Vécsey-Semjén, 1999).

(ii) The family of toxins that enzymatically hydrolyze the phospholipids of the bilayer membrane of eucaryotic cells. This family comprises hemolytic and sometimes lethal phospholipases C, certain of them being zinc metallophospolipases. Other toxins exhibit sphingomyelinase or phospholipase D activities (Titball, 1999; and Chapter 27 in this volume). *C. perfringens* α-toxin (phospholipase C), *S. aureus* β-toxin (sphingomyelinase C), and *Vibrio damsela* hemolysin (phospholipase D) are among the most important members of this family. From a historical point of view, the first bacterial protein toxin whose molecular mechanism of action (phospholipase activity) was identified is *C. perfringens* α-toxin (Mac Farlane and Knight, 1941).

(iii) The family of the cytolytic pore-forming toxins (PFTs), which constitutes the majority of the members of MDTs' superfamily, characterized by its property to create channels (pores) through the cytoplasmic bilayer membrane (7–9 nm) of host's cells.

The PFTs family comprises roughly 85 proteins, whereas the enzymatic and tensio-active cytolysins comprise 27 and 10 proteins, respectively.

## THE CONCEPT OF TOXIN (PROTEIN)-INDUCED TRANSMEMBRANE PORES

This concept concerns a general process of disorganization of cytoplasmic cell membrane elicited by a great number of proteins of either procaryotic origin (bacterial toxins) or from eucaryotic organisms (complement, T lymphocytes, perforin, animal venoms, and toxins). This process involves the formation of hydrophilic pores (channels) in the cytoplasmic membrane of target cells provoked by the insertion of these proteins into the membrane, followed by the impairment of cell permeability and thereby cell destruction (lysis). Historically, the first proposal that proteins may create pores in cell membranes concerns the mechanism of erythrocyte lysis by insertion of the C5–C9 components ("membrane complex attack") of plasma complement (Mayer, 1972; Bhakdi and Tranum-Jansen, 1984).

This process was rapidly extended to an increasing number of cytolytic bacterial protein toxins since the

1980s, according to the following *modus operandi* (van der Goot, 2003).

## Pore formation

The toxins are released by the bacteria as water-soluble, generally monomeric proteins. The monomers diffuse toward the target membrane and bind often with great specificity onto various host cells' surface molecules. The surface-bound monomeric PFTs then encounter with other toxin molecules and undergo a series of remarkable changes. They form non-covalently associated oligomers organized into a ring-like structure called the prepore, at least for certain PFTs (Sellman *et al*., 1997; Heuck *et al*., 2003). A subsequent conformational change leads to an amphipathic state (β-barrel profile) of the oligomers, followed by insertion into the membrane and the formation of protein-lined pores of various sizes, depending on the toxin involved. In these pores, the hydrophobic surfaces of toxin molecules are exposed to the membrane acyl-chains and the hydrophilic sides line up the pore (Freer and Birkbeck, 1982; Bhakdi and Tranum-Jensen, 1988; Bhakdi *et al*., 1996; Parker *et al*., 1996; Bayley, 1997; Gouaux, 1997, 1998; Lesieur *et al*., 1997; Palmer *et al*., 1998; Shatursky *et al*., 1999; Gilbert *et al*., 1999; Menestrina *et al*., 2001; van der Goot *et al*., 2003).

## Toxin-binding sites receptors

The chemical nature of the receptors involved in the binding of toxin monomers on target cell surface are known for only a limited number of toxins:

1. Sugar moieties, such as the glycan core of glycosyl phosphatidyl (GPI)-anchored proteins in the case of aerolysin (Buckley, 1999; Fivaz *et al*., 2001; and Chapter 33 of this volume) and *C. septicum* α-toxin (Gordon *et al*., 1999)
2. Cholesterol for the family of "cholesterol-dependent" cytolysins (Alouf and Palmer, 1999; Heuck *et al*., 2001; Giddings *et al*., 2003; and Chapters 36 to 40 of this volume), and *V. cholerae* cytolysin (Zitzer *et al*., 1997; Palmer, 2004)
3. Human CD59 for the "cholesterol dependent" intermedilysin (Giddings *et al*., 2004)
4. Membrane phospholipids (Elias *et al*., 1966; De Azavedo, Chapter 42 of this volume)

## Electron microscopy of cell damage induced by pore-forming toxins

Since the pioneering electron microscopic studies of Dourmashkin and Rosse (1966) on erythrocyte damage by complement and streptolysin O, several investigations on PFTs have been reported. These authors and many others observed at the surface of target cells ring- and arc-shaped structures elicited by various cholesterol-dependent toxins, particularly streptolysin O, pneumolysin, listeriolysin O, and perfringolysin O (Bernheimer and Rudy, 1986; Bhakdi and Tranum-Jensen, 1988; Alouf and Palmer, 1999; Sekiya *et al*., 1993, 1996; Gilbert *et al*., 1999; Vazquez-Boland, 2001). Similar structures were obtained with *E. coli* α-hemolysin (Vandana *et al*., 1997). These structures reflect the oligomerization of the toxins and the formation of conducting pores at the surface of target cells (Gilbert *et al*., 1998, 1999).

## Sizes of the toxin-induced pores

The pores elicited by the toxins differ widely in size. The smallest pores formed in mammalian cell membranes have been sized to around 1–3 nm effective diameter. This is the case for *S. aureus* alpha-toxin (Bhakdi and Tranum-Jensen, 1988; Valeva *et al*., 1997; Fivaz *et al*., 2000), *E. coli* alpha-hemolysin (Lesieur *et al*., 1997; Menestrina and Vécsey-Semjén, 1999), and ClyA cytolytic toxin (Oscarsson *et al*., 1999), *Aeromonas* aerolysin (Buckley, 1999; van der Goot, 2003; Fivaz *et al*., 2001), and *Vibrio* El Tor hemolysin (Zitzer *et al*., 1997). The pores formed by streptolysin S are very small in diameter (Elias *et al*., 1966; Thelestam and Möllby, 1979). In contrast, the transmembrane pores formed by the cholesterol-dependent toxins create holes up to 35–40 nm in diameter corresponding to about 50 monomers of toxin molecules (Sekiya *et al*., 1993, 1996; Gilbert *et al*., 1999; Vazquez-Boland, 2001).

## Extension of the classical pore-forming concept

The classical concept concerns those soluble PFTs molecules that oligomerize at the surface of target cell and punch holes through the bilayer cytoplasmic membrane.

However, in certain cases (invading bacteria), other processes take place. Pores can also be generated once the bacterium has invaded the host cell (van der Goot, 2003). This is the case with listeriolysin O and ivanolysin O produced by the intracellular pathogens, *Listeria monocytogenes* and *Listeria ivanovii*, which grow and release these toxins inside the phagocytic vacuole (phagosome). These cytolysins provoke pore formation in the phagosomal membrane, thereby allowing the release of the bacteria in target cell cytosol (Dramsi and Cossart, 2002). In another situation observed with *Legionella pneumophila*, the production of IcmS by this intracellular pathogen leads to pore formation in the

host cell membrane from the cytoplasmic side, allowing local rupture and egress of the bacterium into the cell surroundigs (Burns, 2003; Molmeret *et al*., 2004). Interestingly, other non-cytolytic pore-forming protein toxins oligomerize to form pores as shown for the protective antigen (PA) of *Bacillus anthracis*, which forms heptamers in target cells (Petosa *et al*., 1997). The role of PA is to translocate the enzymatic moieties of anthrax toxin into cell cytosol.

## TYPOLOGY OF PORE-FORMING TOXINS

The cloning and sequencing of the majority of the structural genes of PFTs over the past 15 years, as well as the great progress of our knowledge of the mechanism of action of these cytolysins during the same period, allows a rational classification of these cytolysins into "congeneric" and "individualistic" toxins categories as initially suggested by Bernheimer and Rudy (1986) (Table 26.1). The former category comprises those PFTs that share close structural and (or) similar physicochemical and functional lytic properties and mechanisms of action, while the latter concerns PFTs exhibiting an appreciable degree of individuality or uniqueness.

### Congenering pore-forming toxins

Most PFTs that belong to this category of cytolysins are specifically produced by either Gram-positive or Gram-negative bacteria. However, certain congeneric toxins are produced by both *Clostridum septicum*

**TABLE 26.1** Representative congeneric pore-forming bacterial toxins sharing functional and (or) structural features

| |
|---|
| **Toxins from Gram-positive bacteria** |
| 1. The family of the cholesterol-dependent cytolysins (formerly SH-"activated" toxins):<br>22 toxins produced by 24 bacterial species from the genera: *Streptococcuss (4 species), Bacillus (6 species), Brevibacillus (1 species), Paenibacillus (1 species), Clostridium (9 species), Listeria (3 species), Arcanobacterium (1 species)* (Alouf, 2001; and Chapter 36 of this volume)<br>Eucaryotic organism-related toxin metridiolysin from the sea anemone *Metridium senile* |
| 2. The family of *S aureus* α-helix (α-toxin) and β-barrel pore-forming bicomponent leucocidins and γ-hemolysins sharing sequence homology: The bicomponent entities include 6 classes of S proteins and 5 classes of F proteins: LukS-PV / Luk F-PV / LukS-R / LukF-R / Luk-E / Luk-D/Luk-M /Luk-F'HlgA HlgB/ HlgC), proteins with 30 possible binary combinations (Prévost *et al*., 2001). The hemolytic γ-toxin comprises three components (Comai *et al*., 2002). *S. aureus* α-toxin and the bicomponent leucocidins are distant in sequence, but are similar in structure (Gouaux 1997). *S. intermedius* leucocidins S-I/F-I (Menestrina *et al*., 2001). |
| 3. *C. perfringens* β-toxin, *B. cereus* hemolysin II, and cytotoxin K (Hunter *et al*., 1993; Baida *et al*., 1999; Prévost *et al*., 2001). |
| 4. *S. aureus/S. epidermidis/S. lugdunensis* **δ**-toxin and many similar cytolytic amphipatic animal peptides: melittin (bee venom), mastoparans, etc. *(*Alouf *et al., 1989;* Dufourcq *et al., 1999).* |
| 5. Streptolysin S (SLS) and SLS-like cytolysins (Alouf and Loridan, 1988; Nizet *et al*., 2000; and Chapter 42 of this volume). |
| **Toxins from Gram-negative bacteria** |
| 1. The multigenic encoded RTX *(Repeats in Toxins)* family:<br>At least 27 hemolysins, leucocidins, enterohemolysins, and metalloproteases produced by 23 bacterial species from the genera *Escherichia, Proteus, Morganella, Pasteurella, Mannheimeia, Actinobacillus, Vibrio, Bordetella, Moraxella*, and *Enterobacter* (Welch, 2001; and Chapters 29 and 30 of this volume). |
| 2. The bicomponent A/B cytolysins from: *Serratia, Proteus, Edwardsiella, Haemophilus* species (Hertle, 2002; and Chapter 31 of this volume): Shl B/A (*S. marcescens)*, Hpm B/ A (*P. mirabilis, P. vulgaris)* Eth B/A *(E. tarda)*, Hhd B/A (*H. ducreyi*): Short sequence homologies of either A or B moieties with: *Bordetella pertussis,* filamentous hemagglutinin, *H influenzae,* hemopexin, and surface protein H. |
| 3. The family of cytolysins and cytotoxins of *V. cholerae* and other *Vibrio* species:<br>15 hemolysins related to El Tor and Nag hemolysins, Vmh (*V. mimicus*) and other species, hemolysins related to *V. parahaemolyticus,* and other hemolysins from four species (Zitzer *et al*, 1997; and Chapter 44 of this volume). |
| 4. The family of the enterobacterial cytolytic protein ClyA produced as outer membranes' vesicles by pathogenic *E. coli* and *Salmonella enterica* serovars Typhi and Parathyphi A (Wai *et al*., 2003; Oscarsson *et al*., 2002). |
| **Toxins from both Gram-negative and Gram-positive bacteria** |
| The family of *Aeromonas hydrophila, A. sobria* channel-forming toxins proaerolysin/aerolysin and *Clostridiun septicum* α-toxin (Fivaz *et al*., 2001). |
| Eucaryotic organisms' homologue of the plant cytolytic protein enterolobin (40% sequence identity) from the seeds of *Enterolobium contortisiliquum* (Fontes *et al*., 1997). The small lobe of aerolysin is almost identical to a fold in the S2 and S3 subunits of pertussis toxin. |

α-toxin and (*Aeromonas hydrophila* aerolysin) Ballard *et al*., 1995). The main features of congeneric PFTs are summarized in Table 26.1. For detailed data about these cytolysins, we refer the reader to the relevant specific chapters (Section III of this volume).

### "Individualistic" pore-forming toxins

Among many other PFTs, this wide group comprises *Enterococcus faecalis* and *Bacillus cereus* bi- and tri-component toxins, *Gardnerella vaginalis* toxin, *Legionella pneumophila* legiolysin, *Pseudomonas aeruginosa* leucotoxin (Xiong *et al*., 1994; Bhakdi *et al*., 1996; Menestrina and Vécsey-Semjén, 1999).

## CONCLUSION

Our knowledge on pore-forming toxins and more generally on other classes of membrane-damaging toxins has progressed considerably over the past decade as concerns their structure, genomic aspects, and structure-activity relationships. The study of the biological effects at the subcellular levels and the pathophysiological properties of these toxins also have witnessed remarkable developments. The involvement of some of them in the pathogenesis of various diseases is currently well established. However, the role of many other PFTs in acute and chronic infections remains to be elucidated.

## REFERENCES


Alexander, J.E., Berry, A.M., Paton, J.C., Rubins, J.B., Andrew, P.W. and Mitchell, T.J. (1998). Amino acid changes affecting the activity of pneumolysin alter the behavior of pneumococci in pneumonia. *Microb. Pathog*. **24,** 167–174.

Alouf, J.E. (1977). Cell membranes and cytolytic bacterial protein toxins. In: *Specificity and Action of Animal Bacterial and Plant Protein Toxins* pp. (ed. P. Cuatrecasas), pp. 219–270. Chapman and Hall, London.

Alouf, J.E. (1980). Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Therap*. **11,** 661–717.

Alouf, J.E, Geoffroy, C., Pattus, F. and Verger R (1984). Surface properties of bacterial sulfhydryl-activated cytolytic toxins. Interaction with monomolecular films of phosphatidylcholine and various sterols. *Eur. J. Biochem*. **141,** 205–210.

Alouf, J.E, Dufourcq, J., Siffert, O., Thiaudière, E. and Geoffroy, C. (1989). Interaction of staphylococcal delta-toxin and synthetic analogues with erythrocytes and phospholipid vesicles. Biological and physical properties of the amphipathic peptides. *Eur. J. Biochem*. **183,** 381–390.

Alouf, J.E and Palmer, M. (1999). Streptolysin O. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds . J E. Alouf and J.H. Freer) pp. 459–473. Academic Press, London.

Alouf, J.E. (2001). Pore-forming toxins. In: *Pore-Forming Toxins* (ed. F.G. van der Goot), pp. 1–14. Springer, Berlin.

Arbuthnott (1970). Staphylococcal α-toxin In: *Microbial Toxins*, eds. T.C. Montie, S. Kadis, S. Ajl pp. 189–236. Academic Press, New York.

Arbuthnott J.P. (1982). Bacterial cytolysins (membranes damaging toxins). In: *Molecular Action of Toxins and Viruses*, eds. L. Cohen and S. van Heyningen, New York , Elsevier, 107–109.

Baida, G., Budarina, Z.I., Kuzmin N.P. and Solonin, A.S. (1999). Complete nucleotide sequence and molecular characterization of hemolysin II gene from *Bacillus cereus*. *FEMS Microbiol. Lett*. **180,** 7–14.

Ballard, J., Crabtree, J., Roe, B.A. and Tweten, R. (1995). The primary structure of *Clostridium septicum* alpha toxin exhibits similitary with *Aereomonas hydrophila* aerolysin. *Infect. Immun*. **63,** 340–344.

Bayley, H. (1997). Toxin structure: part of a hole? *Curr. Biol*. **7:** R763–767.

Bernheimer, A.W. (1970). Cytolytic toxins of bacteria. In: *Microbial toxins* (eds. S Ajl, S. Kadis and T.C. Montie), vol. 1, pp. 183–212. Academic Press, New York.

Bernheimer, A.W. (1996). Some aspects of the history of membrane-damaging toxins. *Med. Microbiol. Immunol*. **185,** 59–63.

Bernheimer, A. W. and Rudy, B. (1986). Interactions between membranes and cytolytic peptides. *Biochim. Biophys. Acta*. **864,** 123–141.

Bhakdi, S. and Tranum-Jensen, J. (1984). Mechanism of complement cytolysis and the concept of channel-forming proteins. *Philos. Trans. R. Soc. London ser B* **306,** 311–324.

Bhakdi S. and Tranum-Jensen, J.(1988). Damage to cell membranes by pore-forming bacterial cytolysins. *Prog. Allergy* **40,** 1–43.

Bhakdi, S., Muhly, M., Korom, S. and Hugo, F. (1989). Release of interleukin-1 beta associated with potent cytocidal action of staphylococcal alpha-toxin on human monocytes. *Infect. Immun*. **57,** 3512–3519.

Bhakdi, S. and Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev*. **55,** 733–751.

Bhakdi, S., Bayley, H., Valeva, A., Walev, I., Walker, B., Kehoe, M. and Palmer, M. (1996). Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins. *Arch. Microbiol*. **165,** 73–79.

Billington, S.J., Jost, B.H. and Songer, J.G. (2000). Thiol-activated cytolysins: structure, function, and role in pathogenesis. *FEMS Microbiol. Lett*. **182,** 197–205.

Burns, D. (2003). Type IV secretion systems. In: *Bacterial Protein Toxins* (eds. D. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), pp. 115–187. ASM Press, Washington, D.C.

Bryant, A.E, Chen, R.Y.Z., Nagata, Y., *et al*. (2000a). Clostridial gas gangrene I: cellular and molecular mechanisms of microvascular dysfunction induced by exotoxins of *C. perfringens*. *J. Infect. Dis*. **182,** 799–807.

Bryant, A.E, Chen, R.Y.Z., Nagata, Y., *et al*. (2000b). Clostridial gas gangrene II: phospholipase C-induced activation of platelet gpIIb/IIIa mediates vascular occlusion and myonecrosis in *C. perfringens* gas gangrene. *J. Infect. Dis*. **182,** 808–815.

Bryant, A.E., Bayer, C.R., Hayes-Schroer, S.M. and Stevens, D.L. (2003). Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. *J. Infect. Dis*. **187,** 408–417.

Buckley, A.T. (1999). The channel-forming toxin aerolysin. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J E. Alouf and J.H. Freer), pp. 362–372. Academic Press, London.

Calandra, G.B. and Cole, R.M. (1981). Membrane and cytoplasmic location of streptolysin S precursor. *Infect. Immun*. **31,** 386–390.

Comai, M., Della-Serra, M., Coariola, M., Werner, S., Colin, D., Prévost, G. and Menestrina G. (2002). Protein engineering modulates the transport properties and ion selectivity of the pores

formed by staphylococcal γ–hemolysins in lipid membranes. *Mol. Microbiol*. **44,** 1251–1267.

Coote, J. (1996). The RTX toxins of Gram-negative bacterial pathogens modulators of the host immune response. *Rev. Med. Microbiol*. **7,** 53–62.

Czajkowsky, D.M., Hotze, E.M., Shao, Z., and Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane bete-hairpins to the membrane. *EMBO J*. **23,** 3206–3215.

Dobereiner, A., Schmid, A., Ludwig, A., Goebel, W. and Benz, R. (1996). The effects of calcium and other polyvalent cations on channel formation by *Escherichia coli* alpha-hemolysin in red blood cells and lipid bilayer membranes. *Eur. J. Biochem*. **240,** 454–460.

Dourmashkin, R. and Rosse, W.F. (1966). Morphologic changes in the membranes of red blood cells undergoing hemolysis. *Am. J. Med*. **41,** 699–710.

Dramsi, S. and Cossart, P. (2002). Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. *J. Cell. Biol*. **156,** 943–946.

Dufourcq, J., Castano, S. and Talbot, J.C. (1999). Delta-toxin related hemolytic toxins and peptidic analogues. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J E. Alouf and J.H. Freer), pp. 386–401. Academic Press, London.

Duncan, J.L. (1984). Liposomes as membrane models in studies of bacterial toxins. *J. Toxicol. Toxin. Rev*. **3,** 1–51.

Ehrlich, P. (1898). Discussion during Gesellschaft der Charite Arzte. *Berlin. Klin. Wsch*. **35,** 273.

Elias, N., Heller, M. and Ginsburg, I. (1966). Binding of streptolysin S to red blood cell ghosts and ghost lipids. *Isr. J. Med. Sci*. **2,** 302–309.

Ellemor, D.M., Baird, R.N., Awad, M.M., Boyd, R.L., Rood, J.I. and Emmins, J.J. (1999). Use of genetically manipulated strains of *Clostridium perfringens* reveals that both alpha-toxin and theta-toxin are required for vascular leukocytosis to occur in experimental gas gangrene. *Infect. Immun*. **67,** 4902–4907.

Fivaz, M., Abrami, L., Tsitrin, Y. and van der Gott F.G. (2001). Aerolysin from *Aeromonas hydrophila* and related toxins. In: *Pore-Forming Toxins* (ed. F. G. van der Goot), pp. 35–52. Springer, Berlin.

Fontes, W., Sousa, M.V., Aragao, J.B. and Morhy, L. (1997). Determination of the amino acid sequence of the plant cytolysin enterolobin. *Arch. Biochem. Biophys*. **347,** 201–207.

Freer, J.H. and Birkbeck, T.H. (1982). Possible conformation of delta-lysin, a membrane-damaging peptide of *Staphylococcus aureus*. *J. Theor. Biol*. **94,** 535–540.

Freer, J.H. and Arbuthnott, J.P. (1983). Toxins of *Staphylococcus aureus*. *Pharmacol. Ther*. **19,** 55–106

Frey, J. and Kuhnert, P. (2002). RTX in *Pasteurellaceae*. *Int. J. Med. Microbiol*. **292,** 149–158.

Giddings, J.S., Zhao, J., Sims, P.J. and Tweten, R.K. (2004). Human CD 59 is a receptor for the cholesterol–dependent cytolysin intermedilysin. *Nat. Struct. Mol. Biol*. **12,** 1163–1164.

Gilbert, R.J., Rossjohn, J., Parker, M.W., Tweten, R.K., Morgan, P.J., Mitchell, TJ., Errington, N., Rowe, A.J., Andrew, P.W. and Byron, O. (1998). Self-interaction of pneumolysin, the pore-forming protein toxin of *Streptococcus pneumoniae*. *J. Mol. Biol*. **284,** 1223–1237.

Gilbert, R.J., Jimenez, J.L., Chen, S., Tickle, I.J., Rossjohn, J,. Parker, M., Andrew, P.W. and Saibil, H.R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*. *Cell*. **97,** 647–655.

Gordon, V.M., Nelson, K.L., Buckley, J.T.TN, Stevens, V.L., Tweten, R.K., Elwood, P.C. and Leppla, S.H. (1999). Clostridium septicum alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. *J. Biol. Chem*. **274,** 27274–27280.

Gouaux E. (1997). Channel-forming toxins: tales of transformation. *Curr. Opin. Struct. Biol*. **7,** 566–573.

Gouaux, E. (1998). Alpha-hemolysin from *Staphylococcus aureus*: an archetype of beta-barrel, channel-forming toxins. *J. Struct. Biol*. **121,** 110–122.

Grimminger, F., Sibelius, U., Bhakdi, S., Suttorp, N. and Seeger, W. (1991). *Escherichia coli* hemolysin is a potent inductor of phosphoinositide hydrolysis and related metabolic responses in human neutrophils. *J. Clin. Invest*. **88,** 1531–1539.

Hackett, S.P. and Stevens, D.L. (1992). Streptococcal toxic shock syndrome: synthesis of tumor necrosis factor and interleukin-1 by monocytes stimulated with pyrogenic exotoxin A and streptolysin O. *J. Infect. Dis*. **165,** 879–885.

Harshman, S., Boquet, P., Duflot, E., Alouf, J.E., Montecucco, C. and Papini, E. (1989). Staphylococcal alpha-toxin: a study of membrane penetration and pore formation. *J. Biol. Chem*. **284,** 14978–14984.

Henderson, B., Wilson, M. and Wren, B. (1997). Are bacterial exotoxins cytokine network regulators? *Trends Microbiol*. **5,** 454–458.

Hertle, R. (2000). Serratia type pore-forming toxins. *Curr. Prot. Pept. Sci*. **1,** 75–89.

Hertle, R. (2002). *Serratia marcescens* hemolysin (ShlA) binds artificial membranes and forms pores in a receptor-independent manner. *J. Membr. Biol*. **189,** 1–14.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2003). Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J. Biol. Chem*. **278,** 31218–31225.

Hunter, S.E.C., Brown, G.J.E., Oyston, P.C.F. and Titball, R.W. (1993). Molecular genetics of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma–toxin, and leukocidin of *Staphylococcus aureus*. *Infect. Immun*. **61,** 3958–3965.

Jacobs, T., Darji, A., Weiss, S. and Chakraborty, T. (1999). Listeriolysin the thiol-activated hemolysin of *Listeria monocytogenes*. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer) pp. 511–521. Academic Press, London.

Johnson, M.K. (1977). Cellular location of pneumolysin. *FEMS Microbiol. Lett*. **2,** 243–245.

Kayal, S., Lilienbaum, A., Poyart, C., Memet, S., Israel, A. and Berche, P. (1999). Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kappa B and up-regulation of adhesion molecules and chemokines. *Mol. Microbiol*. **31,** 1709–1722.

König, B., Köller, M., Prevost, G., Piemont, Y., Alouf, J.E., Schreiner, A. and König, W. (1994a). Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, and erythrogenic toxin A): generation of interleukin-8. *Infect. Immun*. **62,** 4831–4837.

König, B., Ludwig, A., Goebel, W. and König, W. (1994b). Pore formation by the *Escherichia coli* alpha-hemolysin: role for mediator release from human inflammatory cells. *Infect. Immun*. **62,** 4611–4617.

Lally, E.T., Hill, R.B., Kieba, I.R. and Korostoff, J. (1999). The interaction between RTX toxins and target cells. *Trends Microbiol*. **7,** 356–361.

Lai, X.-H., Arencibia, I., Johansson, A., Wai, S.N., Oscarsson, J., Kalfas, S., Sundqvist, K.-G., Mizunoe, Y., Sjösted A. and Uhlin, B.E. (2000). Cytocidal and apoptotic effects of the ClyA protein from *Escherichia coli* on primary and cultured monocytes and macrophages. *Infect. Immun*. **68,** 4363–4367.

Launay, J.M. and Alouf, J.E. (1979). Biochemical and ultrastructural study of the disruption of blood platelets by streptolysin O. *Biochim. Biophys. Acta* **556,** 278–291.

Launay, J.M., Geoffroy, C., Costa, J.L. and Alouf, J.E. (1984). Purified SH-activated toxins (streptolysin O, alveolysin): new tools for

determination of platelet enzyme activities. *Thromb. Res*. **33,** 189–196.

Lesieur, C., Vecsey–Semjn, B., Abrami, L., Fivaz, M. and van der Goot, F.G. (1997). Membrane insertion: the strategy of toxins. *Mol. Membr. Biol*. **14,** 45–64.

Locht, C. (1999). Molecular aspects of *Bordetella pertussis* pathogenesis. *Internatl. Microbiol*. **2,** 137–144.

Lund,T., De Buyser, M.-L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. **38,** 254–261.

Mac Farlane, M.G. and Knight, B.C.J.G. (1941). The biochemistry of bacterial toxins. I. Lecithinase activity of *Cl. welchii* toxins. *Biochem. J*. **35,** 884–902.

Maget-Dana, R. and Peypoux, F. (1994). Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology, **87,** 151–174.

Manteca, C., Daube, G., Jauniaux, T., Linden, A., Pieson, V., Detilleux, J., Ginter, A., Coppe, P., Kaeckenbeeck, A. and Mainil, J.G. (2002). A role for the *Clostridium perfringens* beta2 toxin in bovine enterotoxaemia? *Vet. Microbiol*. **86,** 191–202.

Marmorek, A. (1902). La toxine streptococcique. *Ann. Inst. Pasteur* **16,** 169–178.

May, A.K., Sawyer, R.G., Gleason, T., Whitworth, A. and Pruett, T.L. (1996). *In vivo* cytokine response to *Escherichia coli* alpha-hemolysin determined with genetically engineered hemolytic and nonhemolytic *E. coli* variants. *Infect. Immun*. **64,** 2167–2171.

Mayer, M.M. (1972). Mechanism of cytolysis by complement. *Proc. Natl. Acad. Sci. USA*. **69,** 2954–2958.

Menestrina, G. and Vécsey-Semjén, B. (1999). Biophysical methods and model membranes for the study of bacterial pore-forming toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds J.E. Alouf and J.H. Freer) pp. 287–309. Academic Press, London.

Menestrina, G. (2000). Use of Fourier-transformed infrared spectres copy for secondary structure determination of staphylococcal pore-forming toxins. In: *Bacterial Toxins: Methods and Protocols* (eds. O. Holst, Totowa), pp. 115–132. Humana Press, N.J.

Menestrina, G., Dalla Serra, M. and Prévost, G. (2001). Mode of action of β-barrel pore-forming toxins of the staphylococcal α–hemolysin family. *Toxicon* **39,** 1661–1672.

Molmeret, M., Zink, S.D., Han, L., Abu-Zant, A., Asari, R., Bitar, D.M., and Abu Kwaik, Y. (2004) Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome. *Cell. Microbiol*. **6,** 33–48.

Morgan, P.J., Hyman, S.C., Byron, O., Andrew, P.W., Mitchell, T.J. and Rowe, A.J. (1994). Modeling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. *J. Biol. Chem*. **269,** 25315–25320.

Nakamura, M., Sekino-Suzuki, N., Mitsui, K. and Ohno-Iwashita, Y. (1998). Contribution of tryptophan residues to the structural changes in perfringolysin O during interaction with liposomal membranes. *J. Biochem*. **123,** 1145–1155.

Nelson, K.L., Brodsky, R.A. and Buckley, J.T. (1999). Channels formed by subnanomalar concentrations of the toxin aerolysin trigger apoptosis of T lymphomas. *Cell. Microbiol*. **1,** 69–74.

Nishibori, T., Xiong, H., Kawamura, I., Arakawa, M. and Mitsuyama, M. (1996). Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells. *Infect. Immun*. **64,** 3188–3195.

Oscarsson, J., Mizunoe,Y., Li, L., Lai, X.-H., Wieslander, A. and Uhlin, B.E. (1999). Molecular analysis of the cytolytic protein ClyA (SheA) from *Escherichia coli*. *Mol. Microbiol*. **32,** 1226–1238.

Oscarsson, J., Westermark, M., Löfdahl, S., Olsen, B., Palmgren, H., Mizunoe,Y., Wai, S.N. and Uhlin, B.E. (2002). Characterization of a pore-forming cytotoxin expressed by *Salmonella enterica* serovars Typhi and Parathyphi A. *Infect. Immun*. **70,** 5759–5769.

Oxhamre, C. and Richter-Dahlfors, A. (2003). Membrane-damaging toxins: Family of RTX toxins. In: *Bacterial Protein Toxins* (eds. D. Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), pp. 203–214. ASM Press, Washington, D.C.

Palmer, M., Saweljew, P., Vulicevic, I., Valeva, A., Kehoe, M. and Bhakdi, S. (1996). Membrane-penetrating domain of streptolysin O identified by cysteine scanning mutagenesis. *J. Biol. Chem*. **271,** 26664–26667.

Palmer, M., Harris, J.R., Freytag, C., Kehoe, M. and Bakhdi, S. (1998). Assembly of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. *EMBO J*. **17,** 1598–1605.

Palmer, M. (2004). Cholesterol and the activity of bacterial toxins. *FEMS Microbiol. Lett*. **238,** 281–289.

Paton, J.C. (1996). The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae. Trends in Microbiol*. **4,** 103–106.

Parker, M.W., van der Goot, F.G. and Buckley, J.T. (1996). Aerolysin—the ins and outs of a channel forming toxin. *Mol. Microbiol*. **19,** 205–212.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S. H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* 385, 833–838.

Prévost G., Mourey L., Colin D.A. and Menestrina, G. (2001). Staphylococcal pore-forming toxins In: *Pore-Forming Toxins* (ed. F. G. van der Goot), pp. 53–83. Springer, Berlin.

Rossjohn, J., Gilbert, R.J., Crane, D., Morgan, P.J., Mitchell, T.J., Rowe, A.J., Andrew, P.W., Paton, J.C., Tweten, R.K. and Parker, M.W. (1998). The molecular mechanism of pneumolysin, a virulence factor from Streptococcus pneumoniae. *J. Mol. Biol*. **284,** 449–461.

Rossjohn, J., Tweten, R.K., Rood, J.I., Parker and M.W. (1999). Perfringolysin O. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), pp. 496–510. Academic Press, London.

Rowe, G.E., and Welch, R.A. (1994). Assays of hemolytic toxins. *Meth. Enzymol.* **235,** 657–667.

Rubins, J.B., Charboneau, D., Fasching, C., Berry, A.M., Paton, J.C., Alexander, J.E., Andrew, P.W., Mitchell, T.J. and Janoff, E.N. (1996). Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *Am. J. Respir. Crit. Care Med*. **153,** 1339–1346.

Ruiz, N., Wang, B., Pentland, A., and Caparon, M. (1998) Streptolysin O and adherence synergistically modulate proinflammatory responses of keratinocytes to group A streptococci. *Mol. Microbiol*. **27,** 337–346.

Schmiel, D.H. and Miller, V.L. (1999). Bacterial phospholipases and pathogenesis *Microbes Infect*. **1,** 1103–1112.

Sekiya, K., Satoh, R., Danbara, H. and Futaesaku, Y. (1993). A ring-shaped structure with a crown formed by streptolysin O on the erythrocyte membrane. *J. Bacteriol*. **175,** 5953–5961.

Sekiya, K., Satoh, R., Danbara, H. and Futaesaku, Y. (1996). Electron microscopic evaluation of a two-step theory of pore formation by streptolysin O. *J. Bacteriol*. **178,** 6998–7002.

Sellman, B.R., Kagan, B.L. and Tweten, R.K. (1997). Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. *Mol. Microbiol*. **23,** 551–558.

Shatursky, O., Heuck, A. P., Shepard, L.A., Rossjohn J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Shepard, L.A., Heuck, A.P., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin A. an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. *Biochemistry*. **37,** 14563–14574.

Sheppard, J.D., Jumarie, C., Cooper, D.G. and Laprade, R. (1991). Ionic channel induced by surfactin in planar bilayer membranes. *Biochim. Biophys. Acta* **1064,** 13–23.

Stevens, D.L. and Bryant, A.E. (2002). The role of clostridial toxins in the pathogenesis of gas gangrene. *Clin. Infect. Dis*. **35** (Suppl. 1) S93–S100.

Sibelius, U., Schulz, E.C., Rose, F., Hattar, K., Jacobs, T., Weiss, S., Chakraborty, T., Seeger, W. and Grimminger, F. (1999). Role of *Listeria monocytogenes* exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C in activation of human neutrophils. *Infect. Immun*. **67,** 1125–1130.

Staali, L., Monteil, H. and Colin, D.A. (1998). The staphylococcal pore-forming leukotoxins open Ca2+ channels in the membrane of human polymorphonuclear neutrophils. *J. Membr. Biol*. **162,** 209–216.

Suttorp, N., Fuhrmann, M., Tannert-Otto, S., Grimminger, F. and Bhadki, S. (1993). Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. *J. Exp. Med*. **178,** 337–341.

Tanabe, Y., Xiong, H., Nomura, T., Arakawa, M. and Mitsuyama, M. (1999). Induction of protective T cells against *Listeria monocytogenes* in mice by immunization with a listeriolysin O-negative avirulent strain of bacteria and liposome-encapsulated listeriolysin O. *Infect. Immun*. **67,** 568–575.

Tang, P., Rosenshine, I., Cossart, P. and Finlay, B.B. (1996). Listeriolysin O activates mitogen-activated protein kinase in eucaryotic cells. *Infect. Immun*. **64,** 2359–2361.

Thelestam, M.and Möllby, R. (1979). Classification of microbial, plant, and animal cytolysins based on their membrane-damaging effects on human fibroblasts. *Biochim. Biophys. Acta*. **557,** 156–169.

Titbull, R.W. (1999). Membrane damaging and cytotoxic phospholipasas. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds J.E. Alouf and J.H. Freer), pp. 287–309. Academic Press, London.

Thumbikat, P., Briggs, R.E., Kannan, M.S. and Maheswaran, S.K (2003). Biological effects of two genetically defined leukotoxin mutants of *Mannheimia haemolytica*. *Microb. Pathog*. **34,** 217–226.

Tweten, R.K. (2001). *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. *Vet. Microbiol*. **82,** 1–9.

Valeva, A., Palmer, M. and Bhakdi, S. (1997). Staphylococcal alpha-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. *Biochemistry* **36,** 13298–13304.

van der Goot, F.G. (2000) Plasticity of the transmembrane β-barrel. *Trends Microbiol*. **8,** 89–90.

van der Goot, F.G. (2003). Membrane-damaging toxins: pore formation. In: *Bacterial Protein Toxins* (eds. D.Burns, J.T. Barbieri, B.H. Iglewski and R. Rappuoli), pp. 189–202. ASM Press, Washington, D.C.

Vandana, S., Raje, M. and Krishnasastry, M.V. (1997). The role of the amino terminus in the kinetics and assembly of alpha-hemolysin of *Staphylococcus aureus. J. Biol. Chem*. **272,** 24858–24863.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. and Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev*. **14,** 584–640.

Wai, S.N., Lindmark, B., Söderblom, T., Takade, A., Westermark, M., Oscarsson, J., Jana, J., Richter-Dahlfolrs, A., Mizunoe,Y. and Uhlin, B.E. (2003). Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell*, **115,** 25–35.

Walker, B. and Bayley, H. (1995). Key residues for membrane binding, oligomerization, and pore-forming activity of staphylococcal α-hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. *J. Biol. Chem*. **270,** 23065–23071.

Weiglein, I., Goebel, W., Troppmair, J., Rapp, U.R., Demuth, A. and Kuhn, M. (1997). *Listeria monocytogenes* infection of HeLa cells results in listeriolysin O-mediated transient activation of the Raf-MEK-MAP kinase pathway. *FEMS Microbiol. Lett*. **148,** 189–195.

Xiong, G., Struckmeier, M. and Lutz, F. (1994). Pore-forming *Pseudomonas aeruginosa* cytotoxin. *Toxicology* **87,** 69–83.

Zitzer, A., Palmer, M., Weller, U., Wassenaar, T., Biermann, C., Tranum-Jensen, J. and Bhakdi, S. (1997). Mode of primary binding of target membranes and pore formation induced by *Vibrio cytolysin* (haemolysin). *Eur. J. Biochem*. **247,** 209–216.

CHAPTER

# 27

# Membrane-damaging and cytotoxic phospholipases

*Richard W. Titball and Ajit K. Basak*

## INTRODUCTION

Phospholipases have a special place in the history of bacterial toxinology because the α-toxin (phospholipase C) of *Clostridium perfringens* was the first bacterial toxin shown to have an enzymatic mode of action. This certainly explained the remarkable potency of this class of toxins, and of course since that time many bacterial toxins have been shown to be enzymes. As this chapter will highlight, enzymatic activity alone is not sufficient to confer toxic properties on a phospholipase C. Additionally, nowadays it is less clear what is meant by a toxic phospholipase. The idea that such proteins promote overt damage to the host, often leading to lethality, is still a valid concept. However, increasingly it is apparent that these bacterial enzymes might provoke more subtle but nevertheless detrimental effects on the host. Against this background this chapter considers bacterial phospholipases as toxins in the very broadest sense of the word and considers advances that have been made in understanding the molecular basis of toxicity alongside recent research that has identified new phospholipases that might play roles in disease.

## SUBSTRATES FOR PHOSPHOLIPASES

Phospholipids are ubiquitous in biological systems. They are key components of biological membranes and derivatives of phospholipids such as diacyglycerol and inositol triphosphates that serve as messengers within cells (Exton, 1990). The generalized structure of a glycerophospholipid (Figure 27.1) reveals a polar head group linked via a phosphate group and a glycerol backbone to non-polar hydrocarbon (fatty acyl) tails. Sphingolipids (such as sphingomyelin) contain a modified glycerol backbone, linked to fatty acyl tails. The length and degree of saturation of the hydrocarbon tails varies, and their non-polarity means that phospholipids are sparingly soluble in water. The majority of phospholipid in eukaryotes is found in cell membranes where it can adopt a bilayer configuration, with the tail groups embedded in the membrane and the head group's surface exposed.

Phospholipases hydrolyze phospholipids, and the location of the hydrolyzed bond is used to characterize phospholipases as types $A_1$, $A_2$, B, C, or D (Figure 27.1: $PLA_1$, $PLA_2$, PLB, PLC, or PLD; Möllby, 1978). Intriguingly, phospholipases may display concomitant hydrolytic and synthase activities. For example, the *Pseudomonas aeruginosa* PlcH phospholipase C can also function as a sphingomyelin synthase (Luberto *et al*., 2003). A wide variety of assays can be used to measure phospholipase activity. Colorimetric assays use water-soluble phospholipid derivatives (Kurioka and Matsuda, 1976). Other solution phase assays use phospholipid that has been emulsified with detergents to generate micelles (Waite, 1987) or use lipoproteins such as egg yolk lipoprotein (Waite, 1987). All of these assay systems are useful but reveal little about the ways in which phospholipases interact with membranes. Artificial membranes do allow these studies, but living cell membranes are complex mixtures of different phospholipids and proteins, and are able to repair

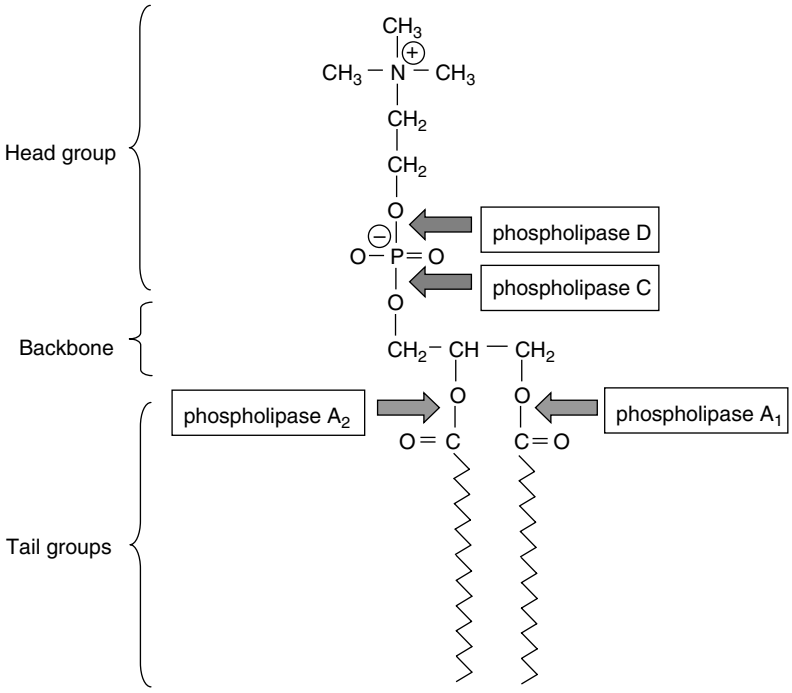



**FIGURE 27.1** Generalized structure of a phospholipid. An entire phosphatidylcholine molecule is shown. In other types of phospholipid, the head group may be inositol, serine, glycerol, or ethanolamine. In sphingomyelins, the backbone includes a $(CH_2)_{12}$. $CH_3$ fatty acyl chain and is termed a ceramide. In all phospholipids, the fatty acyl tails are typically 16–18 carbon chain lengths, but may be shorter or longer and may be unsaturated. The sites of phospholipid cleavage by $PLA_1$, $PLA_2$, PLC, or PLD enzymes are shown arrowed.

limited damage. The lysis of cells, such as erythrocytes, provides a measure of gross cell membrane damage and is easy to perform. However, there is evidence that cellular metabolism is modulated in phospholipase-treated cells and that gross effects on cell membranes (e.g., cell lysis) are only one manifestation of the effect of the enzyme on the cell. These different assay systems are of more than incidental significance: They can reveal the mechanisms by which different enzymes recognize and hydrolyze phospholipids.

## PHOSPHOLIPASES PRODUCED BY BACTERIA

The production of phospholipases by a variety of bacterial species has been reported, many of which are pathogens of man or animals. These enzymes have been grouped and classified as PLA, PLC, or PLD enzymes, based on the site of cleavage and preference for different types of phospholipid. For those proteins whose deduced amino acids sequences are known, the grouping of enzymes according to the above criteria coincides with grouping of enzymes according to amino acid sequence homology. Some of the bacterial phospholipases are lethal toxins, and this chapter considers membrane active bacterial phospholipases, the roles these enzymes play in the pathogenesis of disease, and the molecular basis of membrane interactions.

### Phospholipases A (PLAs)

PLAs are produced by a range of bacteria. Many of these enzymes are membrane bound, and in the case of *Escherichia coli*, they are known as detergent-resistant (DR) phospholipases (Homma *et al*., 1984). This group of PLAs are found in a wide range of Gram-negative bacteria including *Salmonella enterica*, *E. coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Helicobacter pylori* (Dorrell *et al*., 1999), and *Yersinia pseudotuberculosis*. There is no good evidence that these enzymes act as toxins, although the *Y. pseudotuberculosis* does appear to play a role in virulence (Karlyshev *et al*., 2001). A hemolytic cell surface PLA has also been reported from *Legionella pneumophila* (Flieger *et al*., 2004) and a surface bound $PLA_2$ is produced by Campylobacter sp (Istivan *et al*., 2004), but these enzymes do not appear to be closely related to the enterobacteriacae PLAs or to each other. Some bacteria are reported to produce secreted PLAs, and the *Yersinia enterocolitica* YplA (which is distinct from the surface associated PLA) appears to play a role in virulence (Schmiel *et al*., 1998). *L. pneumophila* also produces a secreted lysophospholipase A (Flieger *et al*., 2002), but neither of the *Legionella* enzymes appears to play a role in growth in host cells (Flieger *et al*., 2004). The *Vibrio parahaemolyticus* thermolabile hemolysin is reported to be an extracellular $PLA_2$ (Shinoda *et al*., 1991) and shows sequence similarity to the PLAs produced by *Vibrio cholera* (Fiore

*et al*., 1997) and by the fish pathogen *Vibrio mimicus* (Lee *et al*., 2002). These enzymes are related to the glycerophospholipid-cholesterol acyltransferases (GCAT) produced by *Aeromonas salmonicida* and *Aeromonas hydrophila* (Fiore *et al*., 1997), which can act as $PLA_2$s in the absence of acyl receptors (Thornton *et al*., 1988). The roles of these enzymes in virulence is not fully clarified, but some, like the *V. mimicus* PLA, are toxic towards cultured cells (Lee *et al*., 2002).

## Phospholipases B (PLBs)

There are some reports of PLBs produced by pathogenic bacteria such as *Moraxella bovis* (Farn *et al*., 2001). However, to date, there is no evidence that these enzymes are toxic or that they play roles in virulence.

## Phospholipases C (PLCs)

### *Gram-positive zincmetallophospholipases C*

All PLCs produced by the species *Clostridia*, *Bacillus*, or *Listeria* are zinc-dependent metallo-enzymes with molecular masses in the range 29–43kDa. The best characterized of these enzymes are the *C. perfringens* α-toxin (Titball *et al*., 1989) and the *Bacillus cereus* phosphatidyl choline-PLC (PC-PLC) (Johansen *et al*., 1988). The zincmetallophospholipases C can be divided into two groups: single domain proteins (e.g., *B. cereus* PC-PLC) with approximately 250 amino acids. The other protein (typified by *C. perfringens* α-toxin) possesses an additional carboxy-terminal domain.

Interestingly, *Bacillus anthracis* also possesses a homologue of the PC-PLC encoding gene, which is transcriptionally silent. However, it is possible that this gene is expressed under anaerobic conditions, which might be found in macrophages (Pomerantsev *et al*., 2003). The zincmetallophospholipases C are all active towards phosphatidyl choline, but individual enzymes have different activities towards other phospholipids. For example, some of the enzymes are also active towards sphingomyelin (Titball, 1993).

The crystal structures of *B. cereus* PC-PLC (Hough *et al*., 1989) and *C. perfringens* α-toxin (Naylor *et al*., 1998) both reveal that the active site contains up to three zinc ions. Crystallographic analysis of the *B. cereus* PC-PLC with substrate analogues (Hansen *et al*., 1993) suggests that the zinc ions form part of the substrate-binding pocket. These zinc ions also play a structural role by bridging helices in these proteins, and this might in part explain the high thermal stability of these proteins. The zinc-coordinating residues, identified from the crystal structures of PC-PLC (Hough *et al*., 1989) and α-toxin (Naylor *et al*., 1998), are conserved in all of the other zincmetallophospholipases for which deduced amino acid sequences are available (Titball, 1993), suggesting that these enzymes all have similar active site architectures. Substitution of the zinc-coordinating residues in α-toxin results in proteins that are devoid of PLC activity and are non-hemolytic and non-lethal (Guillouard *et al*., 1996). This finding confirms that the phospholipase activity is essential for all biological activities of α-toxin.

### *Gram-negative zinc-dependent phospholipases C*

PLC produced by *Pseudomonas fluoresecens* (Preuss *et al*., 2001) and *L. pneumophila* (Aragon *et al*., 2002; PlcA) appear to be members of a class of Gram-negative zinc-dependent PLC. These enzymes also require $Ca^{++}$ ions for activity and are not closely related to the Gram-positive zincmetallophospholipase Cs or to the *Pseudomonas aeruginosa* PC-PLCs (see below). These enzymes may also be structurally related to the recently discovered PlcB enzyme from *P. aeruginosa*, which is able to hydrolyze phosphatidylethanolamine and may be a zinc-requiring enzyme (Barker *et al*., 2004).

### *Gram-negative phosphatidyl choline phospholipases C (PC-PLCs)*

The PlcH phospholipase C produced by *P. aeruginosa* is considered to be the prototypic member of a superfamily of bacterial PLC and acid phosphatases (Stonehouse *et al*., 2002). This group of enzymes includes the phospholipases C of *Burkholderia pseudomallei*, *Burkholderia mallei*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, and the acid phosphatase/phospholipase of *Francisella tularensis*. These proteins have molecular weights of 50–80kDa, and unlike the Gram-positive zinc-metallophospholipase C, they are not inhibited by D609 (Stonehouse *et al*., 2002). Several homologous genes have been identified in various other bacterial species. For example, there are two phospholipases in this superfamily in *P. aeruginosa*, typically three in *M tuberculosis*, and four in *B. pseudomallei*. All these enzymes share some sequence homology; they do not have similar properties. The *M. tuberculosis* proteins are truncated at the C-terminus and posses a C-terminal lipid-binding motif. Unlike the *Pseudomonas* enzymes, which are secreted from the cell via the twin arginine translocation system (Voulhoux *et al*., 2001), the *M. tuberculosis* enzymes appear to be bound to the cell surface (Johansen *et al*., 1996). This might explain the contact-dependent hemolytic activity that is associated with some virulent strains of *M. tuberculosis* (Leao *et al*., 1995).

The *P. aeruginosa* PlcH and PlcN enzymes have been studied most intensively. PlcH and PlcN show overall sequence homology of 59% (Ostroff *et al*., 1990), but

differ in their properties. PlcH is hemolytic and is able to hydrolyze phosphatidylcholine and sphingomyelin, whereas PlcN is non-hemolytic and able to hydrolyze phosphatidylcholine and phosphatidylserine. One of the most intriguing features of PlcH, which has not been reported for any of the other Gram-negative PC-PLCs, is the requirement for two chaperone proteins ($PlcR_{1,2}$) for full activity (Stonehouse *et al*., 2002). $PlcR_{1,2}$ appears to play a role in the export of PlcH, and $PlcR_2$ appears to remain complexed with PlcH such that enzyme purified from culture supernate is a 1:1 heterodimer termed $PlcHR_2$ (Stonehouse *et al*., 2002). $PlcR_2$ possesses an EF-hand motif, but paradoxically $Ca^{++}$ ions inhibit the hemolytic activity of PlcH and $PlcHR_2$ (Stonehouse *et al*., 2002). Therefore, it appears that the role of $Ca^{++}$ ions is different for PlcH activation in contrast to Gram-positive zinc metallo-phospholipase Cs, where $Ca^{++}$ ions are essential for the phospholipid recognition.

### *Other phospholipases C*

The relationship of other Gram-negative PLCs to the above enzymes is not known. Many of these enzymes are cell-associated and are produced by intracellular pathogens, although the significance of this relationship awaits clarification. The *L. pneumophila* enzyme ($M_r$ 50-54kD) requires divalent cations, and treatment with EDTA abolishes activity (Baine, 1988). However, the enzyme is not reactivated by treatment with $Zn^{++}$ ions, suggesting that it is not a zinc metallo-phospholipase C. The activity of the enzymes produced by *Ureaplasma urealyticum* (De Silva and Quinn, 1987) or by the oral spirochaetes, such as *Treponema denticola* (Siboo *et al*., 1989), has only been demonstrated using ρNPPC, so it is not possible to conclude whether these PLCs are phosphatidylcholine-preferring enzymes or are sphingomyelinases.

### *Sphingomyelinases C*

Some bacterial phospholipases C show a remarkable specificity for sphingomyelin. *B. cereus* sphingomyelinase (Yamada *et al*., 1988) and *Staphylococcus aureus* sphingomyelinase (β-toxin; Projan *et al*., 1989) show 56% amino acid sequence identity and have molecular masses of approximately 39kD. Both enzymes require $Mg^{++}$ or $Co^{++}$ ions for activity (Ikezawa *et al*., 1986). The sphingomyelinase produced by *Leptospira interrogans* is related to the *B. cereus* and *S. aureus* enzymes, but appears to be produced as a high molecular weight form (63kD), which is processed at the C-terminus to yield a 39kD mature protein (Segers *et al*., 1990). *B. anthracis* also possesses a homologue of the *B. cereus* sphingomyelinase gene (Pomerantsev *et al*., 2003), but like the PC-PLC gene (see above), it is transcriptionally silent. Also like PC-PLC, the gene may be expressed under some conditions (Pomerantsev *et al*., 2003).

### *Phosphatidylinositol-hydrolysing enzymes*

Several bacterial enzymes ($M_r$ ~ 34 kD) that are specific for phosphatidylinositol have been characterized. These type II soluble PI-PLC enzymes (*B. cereus* PI-PLC: Kuppe *et al*., 1989; *Bacillus thuringiensis* PI-PLC: Henner *et al*., 1988; *Listeria monocytogenes* PLC-A: Mengaud *et al*., 1991) possess similar primary structures, and are able to hydrolyze phosphatidylinositol and glucosyl phosphatidylinositol (GPI). It is possible that the *S. aureus* PI-PLC (apparent $M_r$ 20-33kD: Ikezawa, 1986) and the *C. novyi* PI-PLC (apparent $M_r$ 30,000: Ikezawa, 1986) are related to the *Bacillus* and *Listeria* enzymes. Unlike the mammalian enzymes, none of the bacterial PI-PLCs reported to date are metalloenzymes—in fact, $Zn^{++}$, $Mg,^{++}$ or $Ca^{++}$ ions inhibit the activity of the *B. cereus* and *S. aureus* enzymes (Heinz *et al*., 1998; Ikezawa, 1991). Also, unlike mammalian PI-PLCs (type 1 enzymes), the bacterial enzymes are not able to hydrolyze phosphatidylinositol phosphates to generate inositol triphosphate secondary messengers.

Significant insight into the relationship between the structures and functions of the bacterial (*B. cereus* PI-PLC) and mammalian enzymes (rat PI-PLC-δ1) has been obtained by comparing the crystal structures of these proteins (Figure 27.2; Heinz *et al*., 1998). The bacterial enzyme is a single domain that folds as a $\beta\alpha_8$ barrel, with the active site located in an open cleft at the C-terminus (Heinz *et al*., 1998). The crystal structure of the *L. monocytogenes* PLC-A (Moser *et al*., 1997) also reveals a single domain with very similar architecture to the *B. cereus* enzyme—even though these proteins show only 24% sequence identity. The mammalian enzyme also has a $\beta\alpha_8$ barrel domain, which contains the active site, together with a C2-fold domain, an EF-hand domain, and a PH domain (Figure 27.3; Heinz *et al*., 1998). The crystal structures of these proteins also confirm that only the mammalian enzymes bind a single catalytically essential $Ca^{++}$ ion within the active site cleft, and the residues in C2-domain take part in $Ca^{++}$-ion coordination (Heinz *et al*., 1998).

### *Phospholipases D (PLDs)*

The best-studied group of bacterial PLDs includes the enzymes produced by *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans*, and *Arcanobacterium haemolyticum*, which show 64–97% sequence identity (Songer, 1997) and are related to the PLD in the venom of the brown recluse spider (Songer, 1997; Truett and King, 1993). These PLDs are hemolytic only in the

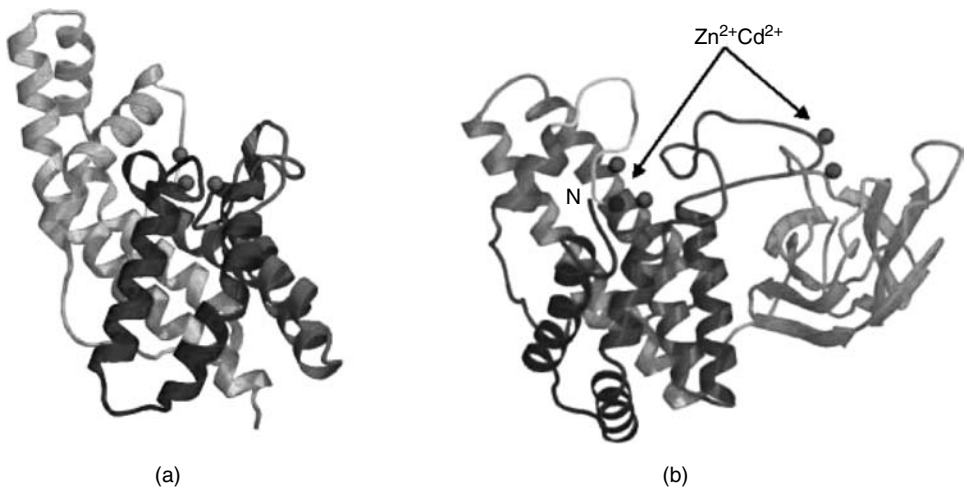



**FIGURE 27.2** Comparison of the crystal structures of the *B. cereus* PC-PLC (a: Hough *et al*., 1989) and the *C. perfringens* α-toxin (b: Naylor *et al*., 1998). Zinc residues found in the active site of the enzymes are shown as spheres. Calcium ions bound to the carboxy-terminal domain of *C. perfringens* α-toxin are also shown as spheres.

presence of the cholesterol oxidase of *Rhodococcus equi*, and pretreatment of erythrocytes with *C. pseudotuberculosis* PLD actually renders the cells resistant to lysis with *C. perfringens* α-toxin or *S. aureus* β-toxin (Soucek *et al*., 1971).

PLDs have also been reported to be produced by a variety of other bacteria, including *Haemophilus influenzae*, *S. enterica*, *Vibrio damsela* (Waite, 1987), *M. tuberculosis* (Johansen *et al*., 1996), *P. aeruginosa* (Wilderman *et al*., 2001), *Yersinia pestis* (Hinnebusch *et al*., 2000), *Neisseria gonorrhoeae* (Edwards *et al*, 2003), and *Rickettsia prowazekii* (Renesto *et al*., 2003). The latter enzyme might account for the activity previously ascribed to a $PLA_2$ produced by the bacterium (Renesto *et al*., 2003). Most of these enzymes have not been well studied. However, the *V. damsela* PLD is hemolytic, preferentially active towards sphingomyelin and able to degrade phospholipids in Madin-Darby canine kidney cells (Kothary and Kreger, 1985). The *R. prowazekii* PLD also appears to be active towards cultured cells, since antibody to PLD is able to reduce the cytotoxicity of the bacterium towards Vero cells (Renesto *et al*., 2003).

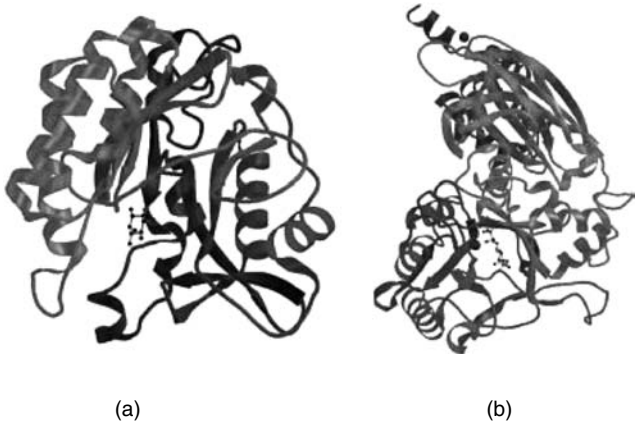



**FIGURE 27.3** Comparison of the crystal structures of the *B. cereus* PI-PLC (a) and rat PI-PLC-δ1 (b). Inositol is shown as a ball and stick molecule in the active site, and $Ca^{++}$ in the active site is shown as a sphere.

## ROLES OF PHOSPHOLIPASES IN DISEASE

In 1944, pioneering work by MacFarlane and Knight showed that the α-toxin produced by *C. perfringens* was an enzyme (MacFarlane and Knight, 1941). This finding stimulated interest in the roles of PLCs produced by other pathogenic bacteria, and even today new and more diverse roles of these enzymes are emerging in the pathogenesis of bacterial disease.

### *Colonization and invasion of host tissues*

Many of the mucosal surfaces of the body are covered by surfactant-like barriers, which are rich in phospholipids, such as phosphatidylcholine. Therefore, it is not surprising that bacterial phospholipases have attracted attention as a possible means of breaching this barrier. The degradation of lung surfactant has been demonstated, *in vitro*, using the α-toxin from *C. perfringens* (Holm *et al*., 1991), and there is no reason to suppose that phospholipases produced by respiratory tract pathogens would not be equally active. It is tempting to speculate that this would enhance the colonization of underlying tissues and while this may be true, the production of phospholipases by pathogens of the respiratory tract play additional roles. The *P. aeruginosa* PLCs are produced *in vivo* in

cystic fibrosis patients (Granström *et al*.,1984), and a PlcN/PlcH mutant was less able than the wild type to induce injury to alveolar epithelial cells (Saiman *et al*., 1992). One function of the *P. aeruginosa* enzymes might be to convert phosphatidylcholine to choline-betaine, which then protects against the high osmotic strength environment in the lung ( Shortridge *et al*., 1992). However, it is studies with the *P. aeruginosa* PlcB that have revealed one of the most remarkable roles of these enzymes. PlcB appears to play a key role in the generation of signaling molecules, such as diacylglycerol, which are then recognized by the bacteria as a chemoattractant (Barker *et al*., 2004). Thus, PlcB may promote the tropism of bacteria towards sites with the greatest potential for colonization. Clearly, the phospholipases of other pathogens of the respiratory tract might play similar roles.

Phospholipases might play similar roles in the case of pathogens that colonize the stomach. For example, the $PLA_2$ produced by *H. pylori* has been suggested to play a role in the degradation of the phosphatidylcholine-rich stomach lining, and thereby allow access to underlying tissues (Langton and Cesareo, 1992). This suggestion is supported by the finding that bismuth salts, which are often used to treat peptic ulcers, are potent inhibitors of the *H. pylori* $PLA_2$ activity (Ottlecz *et al*., 1993). The phospholipases produced by oral spirochaetes might perform similar functions (Siboo *et al*., 1989).

An additional possible role for phospholipases in the invasion of host tissues arises from a recent study with *B. cereus* PC-PLC, which has shown increased matrix metalloprotease production and enhanced permeability of monolayers after treatment of epithelial cell cultures with this enzyme (Firth *et al*., 1997). Whether this mechanism also operates *in vivo* and with PLCs produced by pathogenic bacteria awaits investigation. However, if demonstrated, this process could allow the release of nutrients onto the epithelial cell surface or allow the bacteria to gain access into the body. The *N. gonorrhoeae* PLD also appears to interact with host cells, promoting membrane ruffling and the invasion of cervical epithelial cells (Edwards *et al*, 2003). The molecular basis for this event is not fully clarified, but appears to involve the recruitment of the complement 3 receptor on the cervical cell surface (Edwards *et al*, 2003).

### *Growth and spread of infection in the host*

The production of *B. cereus* or *P. aeruginosa* PC-PLCs is induced under low $P_i$ conditions, suggesting that these enzymes form part of an enzyme cascade involved in phosphate scavenging (Gray *et al*., 1982; Guddal *et al*., 1989). It is worth noting that the PI-PLCs are able to release GPI-anchored proteins, such as alkaline phosphatase and alkaline phosphodiesterase, from the surfaces of eukaryotic cells (Ikezawa, 1986), and it is conceivable that these eukaryotic enzymes also contribute to phosphate scavenging. In addition to their possible role in phosphate acquisition, some phospholipases appear to play a direct role in the spread of infection in the host. Perhaps the most dramatic examples of this are seen with gas gangrene infections in man caused by *C. perfringens* and lymphadenitis or lyphangitis in ruminants or horses caused by *C. pseudotuberculosis*.

The development of gas gangrene is associated with two events. First, a traumatic injury to soft tissues, such as those sustained during road traffic accidents or during warfare that allows the entry of the bacterium (present in the soil or decaying organic matter) into the host. Second, disruption to the blood supply, which might occur following the severing of major blood vessels, results in anoxic conditions in tissues necessary for the growth of *C. perfringens*—an anaerobe. In the mouse model of disease, gas gangrene can be caused by the inoculation of a large dose of bacteria into the hind limb (Awad *et al*., 1995; Williamson and Titball, 1993). The infection progresses rapidly and can be fatal within just 18hr (Awad *et al*., 1995). Since the early 1900s, α-toxin has been suggested to be the major virulence determinant associated with the spread of the infection (McNee and Dunn, 1917). However, there was no evidence until 1995 when a *plc*-mutant of *C. perfringens* was tested in the murine model with little evidence of infection, minimal muscle necrosis, and no deaths of experimental animals (Awad *et al*., 1995).

Several recent studies have revealed the mechanisms by which α-toxin reduces the blood supply to tissues, which then become sufficiently anoxic to allow growth of the bacteria. The α-toxin appears to promote the contraction of the smooth muscle lining blood vessels (Fujii and Sakurai, 1989) and the formation of intravascular aggregates of platelets, leucocytes, and fibrin (Awad *et al*., 2001; Bryant *et al*., 2000). These events would reduce the blood supply to tissues. The formation of the intravascular aggregates appears to involve the up-regulation of a range of cell surface markers, such as platelet gpIIbIIIa (Bryant *et al*., 2003) and β2 integrins on neutrophils (Ochi *et al*., 2002). Initially, these aggregates are free-flowing, but later become bound to endothelial tissues (Bryant *et al*., 2000). Not only do these events reduce the blood supply, but they also modulate the influx of neutrophils into the site of infection (see below).

There is also increasing evidence that some phospholipases play key roles in the establishment of chronic infection. For example, the PLD produced by *C. pseudotuberculosis* appears to be essential for the formation of subcutaneous abscesses in sheep or goats

(Hodgson *et al*., 1992; McNamara *et al*., 1994). In contrast to *C. perfringens* α-toxin, which reduces the blood supply to infected tissues, the *C. pseudotuberculosis* PLD increases vascular permeability. This might allow bacterial spread from the primary sites of infection to the regional lymph nodes where chronic abscesses become established (McNamara *et al*., 1994). The *M. tubeculosis* PLCs appear to play quite a different role in the establishment of chronic disease—possibly because they release fatty acids from host cells as a carbon and energy source in the later stages of infection (Raynaud *et al*., 2002). Consequently, although *M. tuberculosis* PLC mutants are able to grow in macrophages, triple or quadruple mutants show a ten-fold reduction in the colonization of mouse lungs in the later stages of disease (Raynaud *et al*., 2002). This pattern of reduced colonization of lung tissues during the later stages of disease is remarkably similar to that reported for a *pldA* (PLD) mutant of *P. aeruginosa*, raising the possibility that this enzyme plays a similar role to the *M. tuberculosis* PLCs in establishing chronic disease (Wilderman *et al*., 2001).

The PLCs produced by *L. monocytogenes* (PLC-A and PLC-B) play yet a different role in the colonization of host cells by degrading the phagosome membrane and degrading the double membrane vacuole that forms when bacteria spread from cell to cell (Figure 27.4). PLC-A and PLC-B appear to have overlapping but distinct functions. Initial studies with single Δ*plcA* or Δ*plcB* mutants suggested that these enzymes played a minor role in host cell colonization. Further studies have shown that a *ΔplcA ΔplcB* double-mutant was 500-fold attenuated in the murine model of disease (Smith *et al*., 1995) and was markedly less effective in escaping from the phagosome and in spreading from cell to cell (Smith *et al*., 1995).

Other PLCs appear to play less important roles in the infection process. In the mouse model of mastitis, the level of tissue colonization by a β-toxin mutant of *S. aureus* was actually greater than that of the wild type (Bramley *et al*., 1989). While this suggests that β-toxin does not play a role in disease, it should be borne in mind that *S. aureus* causes a wide variety of different diseases in man and animals. It seems unlikely that the bacterium would retain a gene encoding a protein that was not required for survival or growth of the bacterium in any niche. Perhaps the enhanced colonization levels of the β-toxin mutant of *S. aureus* in the mastitis model indicate the role of β-toxin in the development of a stable host-pathogen relationship.

While most of this chapter is devoted to the consideration of the roles of phospholipases in the pathogenesis of diseases of mammalian hosts, it is also worth highlighting the possibility that these enzymes play a role in diseases of non-mammalian hosts. Perhaps the most dramatic example of this is seen in the case of the *P. aeruginosa* PlcH, which not only plays a role in disease of mammalian hosts but also in the growth of the bacterium in plant (*Arabidopsis thaliana*) tissues (Rahme *et al*., 1995). Whether the enzyme exerts similar effects in these markedly different host species awaits investigation.

### *Avoidance of host defense mechanisms*

The cytolytic properties of some PLCs extend to cells other than erythrocytes, and cytolytic activity towards host phagocytes has been reported (Titball *et al*., 1993). There is no evidence that lysis of phagocytic cells occurs on a large scale *in vivo*, but there is good evidence that PLCs can modulate the host response to infection in more subtle ways, thereby allowing the growth of bacteria. The usual host response to infection is to mount a dramatic inflammatory response with the migration of phagocytes into the infected tissues. It has been known for many years that gangrenous infected tissues were devoid of phagocytes (McNee and Dunn, 1917). This pattern is also seen in tissues taken from mice experimentally infected with wild-type *C. perfringens*, but not in tissues taken from mice infected with a Δplc mutant where the expected influx of phagocytes into infected tissues did occur (Stevens *et al*., 1997). In an elegant study, it was shown that α-toxin caused the

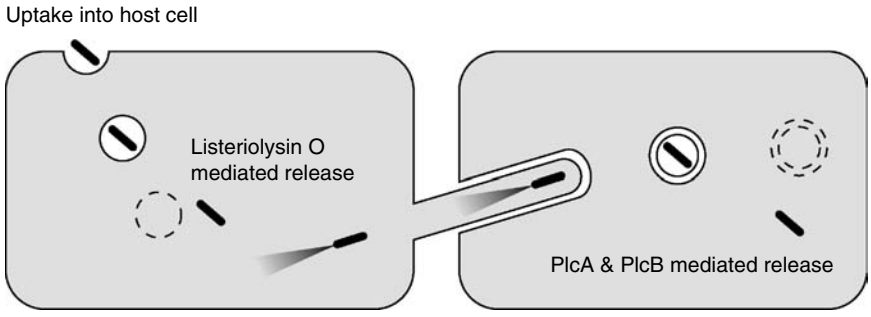



**FIGURE 27.4** Roles of membrane active proteins in the colonization of host cells by *Listeria monocytogenes*. After uptake by host cells, escape from the phagosome is mediated mainly by listeriolysin. Bacteria spread from cell to cell via double-walled vacuoles. Escape from these double-walled vacuoles is mediated mainly by the phospholipases PLC-A and PLC-B. Bacteria with mutations in *PlcA* and *PlcB* are able to infect cells, but are defective in cell-to-cell spread, resulting in small plagues in cell culture.

up-regulation of cell adherence markers (ICAM, ELAM, and p-selectin) on the surface of endothelial cells lining blood vessels (Bunting *et al*., 1997). Neutrophils treated with α-toxin appear to up-regulate β2-integrins, which mediate binding to fibrinogen and fibronectin (Ochi *et al*., 2002), and this might also contribute to neutrophil mistrafficking. Therefore, it appears that α-toxin causes mistrafficking of host phagocytes, allowing the bacterium to grow in host tissues. Whether other bacterial phospholipases also cause host phagocyte mistrafficking awaits investigation.

There are at least two examples where phospholipase play a role in resistance to killing in the host. The *Y. pestis* PLD is also known as the so-called murine toxin, which is reportedly highly toxic for mice. However, paradoxically, this enzyme does not appear to play a role in the pathogenesis of disease in mice (Hinnebusch *et al*., 2000), but rather in the colonization of the flea vector (Hinnebusch *et al*., 2002). The PLA of *H. pylori* appears to be required for survival of the bacterium in the acid conditions in the stomach (Dorrell *et al*.,1999). In both of these cases, the phospholipase appears to play a role in the remodeling of the bacterial membrane to allow the bacterium to survive in the host (Hinnebusch *et al*., 2002; Tannaes *et al*., 2001).

### *Damage to the host*

Early studies with PLCs focused on their cytolytic properties, and there was a general assumption that these enzymes caused widespread cell lysis in the host. Although lysis of erythrocytes does occur in experimental gas gangrene (evidenced as hematuria), there is little evidence that extensive hemolysis occurs during natural cases of gas gangrene or any other diseases involving phospholipase-producing bacteria. Speculatively, one might argue that phospholipases could also contribute to acute respiratory distress syndrome, which is associated with the breakdown of the lung surfactant layer, typically by host phospholipases (Murakami and Kudo, 2004). However, the current evidence indicates that bacterial phospholipases damage the host by subtly perturbing host cell metabolism and in some cases by inducing the production of pro-inflammatory cytokines (Wieland *et al*., 2002).

Many derivatives of phospholipids act as secondary messengers within eukaryotic cells (Exton, 1990). Diacylglycerol, which may be generated after cleavage of glycerophospholipids by phospholipases C, has profound effects on cellular metabolism (Exton,1990). Diacylglcerol lipase can convert diacylglycerol into arachidonic acid, which is then able to enter the arachidonic acid cascade (Samuelsson, 1993), with the resultant generation of prostaglandins, thromboxanes, and leukotrines (Figure 27.5). Treatment of cultured cells or isolated tissues with a variety of phospholipases, including *C. perfringens* α-toxin (Bunting *et al*., 1997; Fujii and Sakurai, 1989) or *P. aeruginosa* Plc-H (König *et al*., 1997), has been shown to result in the generation of these molecules. The diacylglycerol

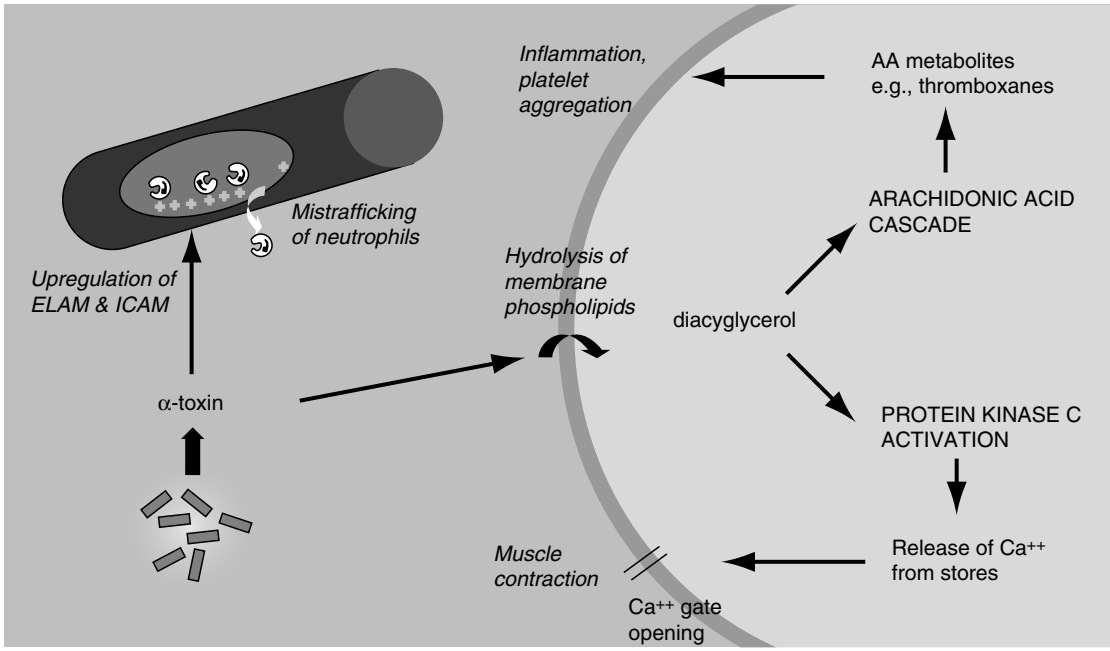



**FIGURE 27.5** Effects of *C. perfringens* α-toxin on mammalian cell metabolism

might also activate endogenous protein kinase C, which is essential for both short- and long-term effects on cellular metabolism (Bunting *et al*., 1997; Nishizuka 1992). The elevated production of matrix metalloproteases by cells treated with *B. cereus* PC-PLC is also thought to be due to activated protein kinase C (Firth *et al*., 1997), and the activation of protein kinase C is also implicated in the up-regulation of cell adherence markers on neutrophils treated with α-toxin (Ochi *et al*., 2002).

The effects of bacterial phospholipases on mammalian cells might be further potentiated as a result of activation of endogenous membrane phospholipases and especially phospholipases $A_2$, C, and D (Gustafson and Tagesson, 1990; Ochi *et al*., 1996; Sakurai *et al*., 1993). In studies with rabbit erythrocyte membranes, it has been shown that endogenous PLC is rapidly activated, followed later by the activation of PLD (Ochi *et al*., 1996; Sakurai *et al*., 1993). These activated mammalian enzymes might themselves generate further substrates for the arachidonic acid cascade. The mechanism by which the mammalian phospholipases are activated has been the subject of speculation. Some workers have suggested that activated protein kinase C is able to activate the mammalian enzymes, either directly or indirectly (Exton, 1990; Nishizuka, 1992; Waite, 1987). An alternative explanation is that the α-toxin mediates the effect via activation of GTP-binding proteins, which in turn activates mammalian phospholipases (Ochi *et al*., 2002) .

In addition to the further activation of the arachidonic acid cascade, mammalian PLCs are able to hydrolyze phosphatidylinositol diphosphate ($PIP_2$) to generate the secondary messenger inositol triphosphate ($IP_3$; Sakurai *et al*., 1993). The $IP_3$ would have a variety of effects on cells. For example, the release of calcium from the endoplasmic reticulum is triggered by $IP_3$, causing the opening of calcium gates in the cell membrane (Fujii *et al*., 1986). Boethius *et al*. (1973) showed that muscle cells treated with α-toxin become inexcitable, and this might explain the cardiotoxic effects of the α-toxin (Asmuth *et al*., 1995). The elevated intracellular calcium levels would also contribute to the activation of endogenous membrane phospholipases described in the previous paragraph.

The effects of α-toxin on eukaryotic cells might be further potentiated by the metabolic status of the cells. Cells deficient in UDP-glucose, a consequence of ischemia, are $10^5$ times more sensitive to *C. perfringens* α-toxin (Flores-Díaz *et al*., 1997). The molecular basis for this enhanced susceptibility has not been fully elucidated. However, UDP-glucose deficient cells contained normal levels of phosphatidylcholine, suggesting that alterations to the outer membrane leaflet were not responsible for the enhanced sensitivity. However, it is apparent that the products of sphingolipid hydrolysis (i.e., ceramide) by sphingomyelinases might also modulate cellular metabolism. Though the abilities of bacterial enzymes to activate these pathways have not been studied in detail, it is perhaps worth pointing out that ceramide is thought to play an important role in the induction of apoptotic responses (Spiegel *et al*., 1996).

In summary, these elegant studies tend to support the hypothesis that the bacterial enzymes are mimicking the effects of the mammalian enzymes and thereby modulating cellular metabolism to the detriment of the host. The effects that have been described above are all relatively short-term. It is also worth mentioning that long-term perturbation of cellular messengers such as protein kinase C has been linked with the transformation of cultured cells. Such effects might suggest a link between the development of cancers and the long-term exposure to bacterial phospholipases (Parkinson *et al*., 1987).

## INTERACTION OF PHOSPHOLIPASES WITH MEMBRANE PHOSPHOLIPIDS

A central feature of phospholipases, which play roles in the pathogenesis of disease, is their ability to interact with membranes, whether from outside the cell or from inside phagosomes. Bacterial phospholipases vary widely in their abilities to interact with membrane phospholipids, and this has resulted in the suggestion that those enzymes that play important roles in the pathogenesis of disease can be identified on the basis of their hemolytic activity. To some extent this proposal is supported by experimental evidence. Of the two related PLCs produced by *P. aeruginosa*, PlcH is hemolytic and able to induce the release of inflammatory mediators from granulocytes, whereas PlcN is devoid of both activities (König *et al*., 1997). A similar pattern is observed when the properties of *C. perfringens* α-toxin are compared with the *Clostridium bifermentans* PLC, *Clostridium sordellii* PLC, or *B. cereus* PC-PLC; the *C. perfringens* α-toxin has markedly higher hemolytic activity than the other enzymes (Jepson *et al*., 1999; Karasawa *et al*., 2003; Titball *et al*., 1991) and is able to cause release of inflammatory mediators from granulocytes (König *et al*., 1997) and is lethal when administered into animals.

The mechanisms that allow some enzymes to interact with membrane phospholipids have been the subject of various studies, and various theories have been developed to explain the mode of action of these

enzymes. One theory proposes that membrane active enzymes have structural features that allow their insertion into membranes. A second theory suggests that membrane active enzymes are able to hydrolyze both phosphatidylcholine and sphingomyelin. These theories are discussed in more detail below.

### *Insertion into the membrane bilayer*

It is generally assumed that the surface of the phospholipid bilayer consists of tightly packed head groups, which mask the hydrophobic tail groups. This model would suggest that the bond susceptible to cleavage by the phospholipase is not exposed to the aqueous phase. In reality, the phospholipids adopt a "hockey stick" conformation in the bilayer, and the susceptible bond might be partially accessible to an enzyme in the aqueous phase (Figure 27.6). However, even with the "hockey stick" conformation of phospholipids, the active site of the enzyme could not be docked with the substrate without the phospholipase either becoming inserted into the membrane or having a mechanism that allowed retraction of the phospholipid out of the membrane. Evidence supporting the "insertion" hypothesis was originally reported by van Deenen *et al.*, (van Deenen *et al.*, 1976), who measured the activity of phospholipases towards phospholipid monolayers maintained at increasing lateral pressures. This showed (Table 27.1) that hemolytic phospholipases (e.g. *C. perfringens* α-toxin) were able to insert into monolayers maintained at lateral pressures above those found in erythrocytes. In contrast, non-hemolytic enzymes (such as *B. cereus* PC-PLC) were only able to hydrolyze phospholipids in monolayers maintained at low lateral pressures.

### *Surface-exposed hydrophobic amino acids might become buried in membranes*

Several regions on the surface of α-toxin have been identified as having possible membrane-binding functions (Naylor *et al.*, 1998). $Trp_{214}$ (in N-terminal domain), $Tyr_{331}$, and $Phe_{334}$ (in C-terminal domain) are surface exposed and are positioned in such a way that they could interact with the phospholipid tail groups on membrane binding (Figure 27.7). In *B. cereus* PC-PLC, $Trp_{214}$ is not exposed; instead, an insertion of a pair of antiparallel helices and $Tyr_{331}$ and $Phe_{334}$ are not present since there is no carboxy-domain in this enzyme. Other bacterial phospholipases might exploit similar mechanisms to allow insertion into the membrane. The *B. cereus* PI-PLC has a "ridge" of hydrophobic amino acids, which are thought to become inserted into the membrane (Heinz *et al.*, 1998). This region also appears to be important in the structurally related *Bacillus thuringiensis* PI-PLC, and two tryptophan residues ($Trp_{47}$ and $Trp_{242}$) have been shown to play a major role in membrane binding (Zhang *et al.*, 2004).

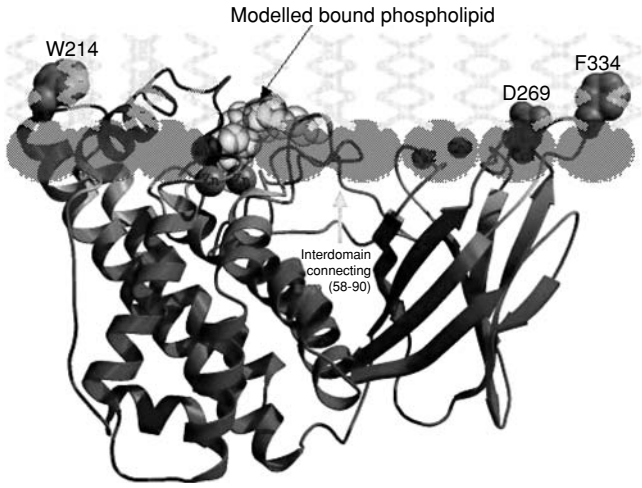



**FIGURE 27.6** Model for the interaction of the *C. perfringens* α-toxin with membrane phospholipids. Reproduced with the permission of Macmillan Magazines Ltd. from Naylor *et al.*, 1998.

**TABLE 27.1** Effect of lateral pressure on the hydrolysis of phospholipids in monolayers by different phospholipases.

| Source of enzyme | Type | Hemolysis of human erythrocytes | Maximum lateral pressure at which hydrolysis ceases (dyne/cm) |
|---|---|---|---|
| Pig pancreas | $A_2$ | – | 16.5 |
| Cabbage | D | – | 20.5 |
| *Crotalus adamanteus* venom | $A_2$ | – | 23 |
| *B. cereus* PC-PLC | C | – | 31 |
| *Naja naja* venom | $A_2$ | + | 34.8 |
| Bee venom | $A_2$ | + | 35.3 |
| *S. aureus* | C | + | >40 |
| *C. perfringens* | C | + | >40 |

Modified from Möllby *et al.* (1978) with the permission of Academic Press Ltd.

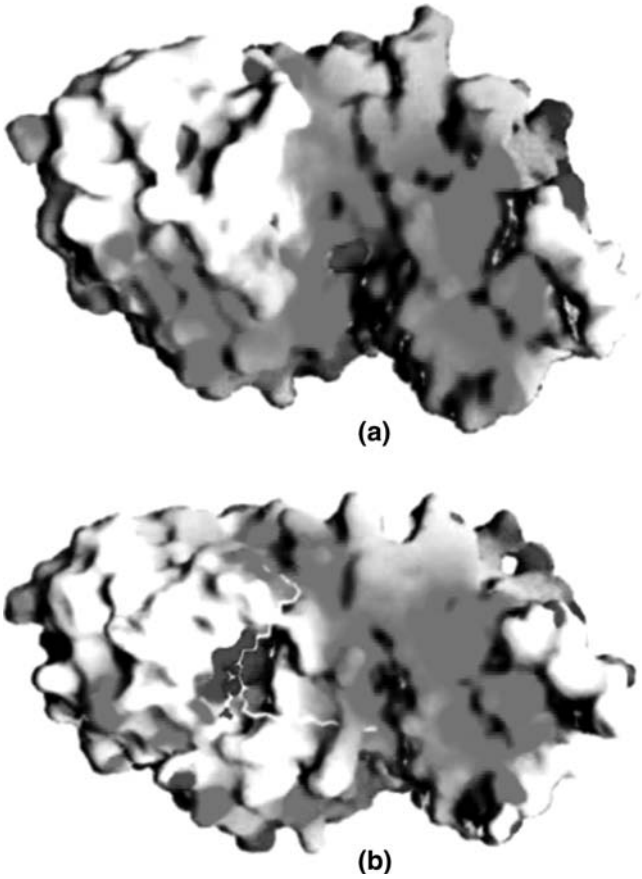



**FIGURE 27.7 GRASP electrostatic surface potential of the closed (a) and open (b) forms of alpha toxins as viewed from where the membrane is thought to bind.** A modeled spingomyelin molecule has been shown in the active site cleft of the open form of the toxin.

### *C2 domains might be involved in phospholipid recognition*

On comparing the structures of *B. cereus* PC-PLC (Hough *et al*., 1989) and *C. perfringens* α-toxin (Naylor *et al*., 1998), it is apparent that both enzymes possess structurally similar catalytic domains, but only *C. perfringens* α-toxin has a carboxy-terminal domain. If this domain is removed from α-toxin, the phospholipase C activity is retained, but hemolytic activity is lost (Titball *et al*., 1991; Nagahama *et al*., 2002). However, the isolated carboxy-terminal domain does not possess hemolytic (or indeed any detectable enzymatic) activity (Titball *et al*., 1993). When the two domains of α-toxin are mixed together in solution, hemolytic activity is restored (Titball *et al*., 1993; Nagahama *et al*., 2002). Even more dramatically, the fusion of the carboxy-terminal domain of α-toxin with the *B. cereus* PC-PLC results in a hemolytic hybrid protein (Nagahama *et al*., 2002). Collectively, these findings confirm that both the catalytic domain and the carboxy-terminal domain are required for membrane activity of the zincmetallophospholipases C.

For many years it has been known that calcium ions are essential for the hemolytic activity of α-toxin (MacFarlane and Knight, 1941) and for the hydrolysis of monomolecular films of phosphatidylcholine (Moreau *et al*, 1988). Three $Ca^{++}$ binding sites have now been identified in the carboxy-terminal domain of α-toxin, and these are located on the putative membrane-binding face of the protein. These $Ca^{++}$ binding sites are organized in a similar way to the calcium-binding sites in eukaryotic C2-fold domains (Naylor *et al*., 1998; Naylor *et al*., 1999). C2-fold domains have not previously been found in prokaryotes, and are typically found in proteins that are involved in lipid metabolism in higher eukaryotes, such as synaptotagmin and phosphatidylinositol-specific phospholipase C (Naylor *et al*., 1998).

The low-binding affinity of $Ca^{++}$ (Kd ~ 175-250 μM at pH 8.0) by the isolated carboxy-terminal domain of α-toxin and the incomplete and/or partial coordination of these ions in $Ca^{++}$ bound structure (Naylor *et al*., 1999) proves that the complete coordination of $Ca^{++}$ ions is partially dependent on membrane phospholipid headgroups (Naylor *et al*., 1999). Variant forms of α-toxin where $Ca^{++}$-binding residues are replaced show reduced activity (Table 27.2), providing further evidence for the role of the carboxy-terminal domain in membrane binding. The binding of $Ca^{++}$ ions by the carboxy-terminal domain appears to be accompanied by structural changes, which expose hydrophobic regions that are otherwise internalized (Naylor *et al*., 1999). This might be consistent with the insertion of these regions into the cell membrane and with the increased fluorescence of acrylodan-labeled α-toxin on binding to liposomes (Nagahama *et al*., 2002).

Does the presence of carboxy-terminal domain provide a simple way of identifying the toxic zincmetallophospholipases C? Unfortunately, the answer is no. The carboxy-terminal domains of the *C. bifermentans* and *C. sordellii* enzymes show approximately 50% sequence identity with the carboxy-terminal domain of *C. perfringens* α-toxin and are therefore likely to be C2-fold domains. Yet these enzymes are only weakly hemolytic and are considered to be non-toxic. To investigate the possibility that these carboxy-terminal domains have different properties, Jepson *et al*. (1999) constructed a hybrid phospholipase C. Removal of the carboxy-terminal domain of the *C. bifermentans* phospholipase C and replacement with the carboxy-terminal domain of α-toxin markedly enhanced hemolytic activity (Jepson *et al*., 1999). The reduced ability of the *C. bifermentans*

**TABLE 27.2** Calcium-binding ligands in the carboxy-terminal domain of *C. perfringens* α-toxin

| Calcium | Coordinating side chains | Mutagenesis | Phenotype of variant toxin compared to wild-type toxin | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $Ca^{++}$ binding | $Ca^{++}$ dependency | Hemolytic activity | PLC activity | Cytotoxic activity | Myotoxic activity |
| Ca1 | $Asp_{269}$ | $Asp_{269}Asn$[a,b] | ND | ND | reduced | ND | reduced | reduced |
| | | $Asp_{269}Tyr$[c] | reduced | ND | increased | reduced | increased | similar |
| | $Gly_{271}$ $Asp_{336}$ $Ala_{337}$ | $Asp_{336}Asn$[b] | ND | ND | reduced | ND | reduced | reduced |
| Ca2 | $Asp_{293}$ $Asn_{294}$ $Gly_{296}$ $Asp_{298}$ | $Asp_{293}Ser$ [d] | reduced | increased | reduced | reduced | reduced | reduced |
| Ca3 | $Thr_{272}$ $Asp_{273}$ $Asn_{297}$ $Asp_{298}$ | $Thr_{272}Pro$[a] | | | | | | |

ND not determined
[a]Guillouard *et al*., 1997
[b]Alape-Giron *et al*., 2000
[c]Jepson *et al*., 2001
[d]Walker *et al*., 2000

protein to interact with membrane appears to be due to the substitution of several key residues identified in α-toxin as playing a key role in membrane binding. For example, the substitution of $Tyr_{331}$ and $Phe_{334}$ in α-toxin with the counterparts in the *C. bifermentans* protein (Leu and Ile, respectively) markedly reduced hemolytic, cytotoxic, and myotoxic activities (Jepson *et al*., 2001). It is likely that other amino acid substitutions on the putative membrane-binding face also contribute to the reduced hemolytic and cytotoxic activity of *C. bifermentans* phospholipase C.

### *The active site may be opened on membrane interaction*

Although the models described previously can explain how phospholipases become partially inserted into the membrane, they do not explain how the enzyme adjusts the position of its active site to make a susceptible bond with the phospholipid. It is possible that the enzymes partially retract the phospholipid from the membrane. Analysis of the structure of the toxic phospholipase C produced by *Clostridium absonum* (structurally related to *C. perfringens* α-toxin) has revealed a hydrophobic channel, which might promote entry of the phospholipid into the active site (Clark *et al*., 2003). Alternatively, the phospholipase may undergo a conformational change that inserts the active site region into the membrane (Blow, 1991). It has previously been suggested that α-toxin might undergo structural changes on activation (Rosenthal and Pousada, 1968), and more recent data indicates that membrane binding is accompanied by structural changes in the protein, which result in the movement of two loops flanking the active site cleft (Naylor *et al*., 1999; Eaton *et al*., 2002). These structural changes may be a consequence of the binding of the carboxy-terminal domain, with the binding event communicated to the active site region via residues 60–90 (Naylor *et al*., 1999). Whatever the precise mechanism by which these structural changes occur, the overall result is the opening of the active site cleft (Eaton *et al*., 2002), and this provides a molecular explanation for long-standing observation of interfacial activation of α-toxin (Klein *et al*, 1975), which results in increased hydrolytic activity towards phospholipids, which are in a membrane-packed form.

## THE MOLECULAR BASIS OF SUBSTRATE SPECIFICITY

### *Substrate specificity and membrane phospholipid hydrolysis*

One of the striking features of the phospholipases is the wide variation in substrate specificity by proteins that

are structurally related. For example, although phosphatidylcholine is the preferred substrate for the zincmetallophospholipases, individual enzymes show differences in their abilities to hydrolyze other phospholipids. The molecular basis for these differences is becoming clearer as crystal structures of these proteins, in some cases complexed with substrate analogs, are becoming available.

One of the key mechanisms by which phospholipases recognize (and discriminate between) their substrates involves the interaction of the head group with the active site. The molecular mechanisms by which phospholipases bind to the head group have been investigated for *B. cereus* PC-PLC (Hanssen *et al*., 1993) and *B. cereus* PI-PLC (Heinz *et al*., 1998). *B. cereus* PC-PLC appears to recognize, primarily, the phosphate moiety that displaces water molecules and becomes coordinated to the three zinc ions in the active site cleft (Hansen *et al*., 1993b). Additional binding occurs via hydrogen bonds between various amino acid side chains and, especially, the choline head group and the carbonyl group (El-Sayed *et al*., 1985; Hansen *et al*., 1993). Remarkably, the substrate preference of the enzyme can be modified by replacing selected amino acids in the headgroup binding pocket (Antikainen *et al*., 2003). The active site of *B. cereus* PI-PLC is relatively wide, to allow entry of the bulky inositol head group, which is recognized primarily via the 4- and 5-hydroxyl groups and various polar and charged side chains of amino acids. In fact, the close interactions between these hydroxyl groups and the active site explain why the enzyme is not able to cleave inositol bisphosphate, since the additional phosphate groups cannot be accommodated within the active site cleft (Heinz *et al*., 1998).

The importance of the recognition of the fatty acyl tails of phospholipids is more variable between different bacterial phospholipases. The recognition of substrate by the bacterial PI-PLCs does not appear to be dependent on recognition of the fatty acyl tail groups (Bruznik *et al*., 1992). In contrast, the fatty acyl tails are recognized by zincmetallophopholipases, such as *B. cereus* PC-PLC and *C. perfringens* α-toxin. The fatty acyl tails must be at least $C_6$ in length for recognition by *B. cereus* PC-PLC (El-Sayed *et al*., 1985), and the recognition of phospholipids in liposomes by *C. perfringens* α-toxin is dependent on the presence of unsaturated fatty acyl tail groups, which are $C_{14}$ or less in length (Nagahama *et al*., 1996). Some of these apparent substrate recognition events might reflect changes in membrane fluidity, especially when liposome assay systems are used. However, it is clear that for some enzymes, recognition of this region of phospholipids is a key event. Since the head groups of sphingomyelin and phosphatidylcholine are usually identical (i.e., phosphorylcholine), the discrimation of these molecules must be dependent on recognition of the glycerol backbone and fatty acyl tail.

For some years it has been suggested that hemolytic phospholipases C were able to hydrolyze both phosphatidylcholine and sphingomyelin, which are major components of the outer leaflet of mammalian cell membranes, whereas non-hemolytic enzymes were able to hydrolyze only one type of phospholipid (Ostroff *et al*., 1990; Titball, 1993). For example, the hemolytic *P. aeruginosa* Plc-H enzyme can hydrolyze both phosphatidylcholine and sphingomyelin, while the related *P. aeruginosa* Plc-N enzyme is able to hydrolyze only phosphatidylcholine and is non-hemolytic (Ostroff *et al*., 1990).

One key difference between *B. cereus* PC-PLC and *C. perfringens* α-toxin is the ability of the latter to hydrolyze sphingomyelin and phosphatidylcholine (Krug and Kent, 1984) whereas the *B. cereus* enzyme is able to hydrolyze only phosphatidylcholine (Otnaess *et al*., 1977). Significantly, a mixture of *B. cereus* PC-PLC and *B. cereus* sphingomyelinase does cause hemolysis, and this mixture is termed cereolysin A-B (Gilmore *et al*., 1989 ; Beecher *et al*., 2000). Why is *B. cereus* PC-PLC active against erythrocyte membranes when combined with a sphingomyelinase, when it is apparently not able to insert into membrane bilayers? It is thought that in membranes, sphingomyelin can sterically obscure the phosphatidylcholine head group (Beecher *et al*., 2000). Therefore, one possibility is that the sphingomyelinase enzyme first hydrolyzes sphingomyelin, and that the resultant changes in membrane organization and lateral pressure allow the phosphatidylcholine hydrolyzing enzyme to act on membrane phospholipids (Beecher *et al*., 2000). Experimental evidence does lend some support to this suggestion. Sphingomyelin plays a key structural role in the outer leaflet of membranes, and erythrocytes incubated with *B. cereus* (or *S. aureus*) sphingomyelinase are not lysed unless they are subsequently exposed to an additional stress, such as cooling below the phase transition temperature (termed "hot-cold lysis") or treatment with divalent cation chelating agents (Bernheimer *et al*., 1974; Ikezawa *et al*., 1986). Viewed together, these findings tend to confirm that when sphingomyelin and phosphatidylcholine are hydrolyzed, the cell membrane is sufficiently weakened, which results in lysis.

### *Membrane-active enzymes may be non-hemolytic*

The above findings suggest that hemolytic activity is indicative of the hydrolysis of both sphingomyelin and phosphatidylcholine, and that hemolytic enzymes (or mixtures of enzymes) are able to penetrate membrane

bilayers. Hemolysis is certainly an extreme measure of cell membrane damage and remains a useful indicator of the interaction of an enzyme with mammalian cells. However, it should not be viewed as the sole indicator of membrane phospholipid hydrolysis. As noted above, bacterial sphingomyelinases are able to hydrolyze membrane sphingomyelin without cell lysis. In other cases non-hemolytic enzymes might act synergistically with other bacterial products to cause hemolysis. One example of this synergy is seen with the phospholipases C produced by *L. monocytogenes*. Although PLC-A and PLC-B are considered to be non-hemolytic (or at least very weakly hemolytic: Geoffroy *et al*., 1991), they play key roles in disruption of the phagosome and the double-membrane vacuole formed during cell-to-cell spread (Smith *et al*., 1995). It is significant that these phospholipases appear to act together, and with the pore-forming toxin listeriolysin O, to achieve these effects (Goldfine *et al*., 1995; Smith *et al*., 1995).

## VACCINES AND THERAPEUTIC APPLICATIONS OF PHOSPHOLIPASES

### Membrane probes

For many years phospholipases have been used as membrane probes—either to specifically hydrolyze different classes of phospholipids in membranes or to mimic the effects of eukaryotic phospholipases C. In the past, the use of phospholipases C in this way has been fraught with many problems; the enzymes were isolated from bacteria, which often produced other membrane-active toxins, and the difficulties of isolating "pure" phospholipases C meant that it was difficult to be certain that the effects observed were due to the bacterial phospholipases C. The availability of cloned gene products means that better defined preparations of phospholipases are now available to support these studies.

### Vaccines

Given the key roles that some phospholipases play in the pathogenesis of disease, it is not surprising that inactivated forms of the enzymes have been considered as components of vaccines. In this respect, *C. perfringens* α-toxin has attracted the most attention, but the toxic nature of this enzyme meant that early vaccination studies used formaldehyde toxoids, prepared from α-toxin isolated from the supernatant fluid of cultures of *C. perfringens* (Boyd *et al*., 1972; Kameyama *et al*., 1975). These toxoids induced protection against gas gangrene, but it was difficult to ensure that the vaccine did not contain other toxoided *C. perfringens* proteins, and therefore to be sure that the protective component in the vaccine was α-toxoid. Several approaches for the generation of genetically engineered toxoids have been reported. Either naturally occurring or laboratory-generated forms of the α-toxin have been reported with markedly reduced enzymatic and cytolytic activity (Guillouard *et al*., 1996; Schoepe *et al*., 2001). Immunization of mice with a naturally occurring non-toxic variant induced protection against subsequent challenge with wild-type α-toxin. These mutated proteins could, in principle, be developed as vaccines if reversion to toxicity were an unlikely event. Alternatively, the recombinant carboxy-terminal domain of α-toxin could be used as a non-toxic vaccine. Immunization of mice with this protein induced high levels of antibody, which protected mice against experimental gas gangrene caused by *C. perfringens* (Williamson and Titball, 1993).

The finding that the *C. pseudotuberculosis* PLD plays a key role in the pathogenesis of caseous lymphadenitis in sheep and goats has prompted suggestions that either a PLD mutant might itself be used as a vaccine (Hodgson *et al*., 1992) or that an enzymatically inactive form of the PLD might be used as a component of a subunit vaccine (Haynes *et al*., 1992).

The possibility that phospholipases produced by other bacteria might be used as components of vaccines has yet to be investigated. However, it should be borne in mind that in the case of intracellular pathogens, it may be difficult to induce an appropriate protective immune response. Remarkably, phospholipases C have also been exploited to solve this problem. In trials in humans, *L. monocytogenes* strains with mutations in the *actA* and *plcB* genes appear to be safe vaccine vectors, which might promote the induction of cellular responses to heterologous antigens (Angelakopoulos *et al*., 2002).

### Design of therapeutics

When bacterial phospholipases play key roles in the pathogenesis of disease, it is feasible that enzyme inhibitors could be used to treat these diseases. For example, bismuth salts, which can be used to treat gastric and duodenal ulcers, are thought to inactivate the *H. pylori* $PLA_2$, and it may be possible to design more effective enzyme inhibitors. The possibility that prokaryotic and eukaryotic phospholipases have similar structures or molecular modes of action suggests that the bacterial enzymes might be used to design and test drugs for use against eukaryotic phospholipases C or lipid-binding enzymes. Such drugs might be used to treat a variety of

inflammatory diseases in man. In this respect, it is interesting to note that the C-terminal domain of the *C. perfringens* α-toxin is folded as a C2-domain, which is found frequently in eukayotic enzymes, such as synaptotagmin, phosphatidylinositol PLC, and arachidonate-5-lipoxygenase (Naylor *et al*., 1998).

## Future directions for research

For many years, studies with bacterial phospholipases were limited to the enzymes produced by *C. perfringens* and *B. cereus*. More recently, studies have been carried out with phospholipases produced by other pathogens. However, there are still many phospholipases produced by pathogens that are poorly characterized and for which we have not determined their roles in the pathogenesis of disease. Why are some enzymes membrane bound and others exported from the cell, and, more generally, are the enzymes produced by intracellular pathogens specially adapted for this lifestyle?

In general, the interaction of phospholipases with membrane phospholipids is thought to be a key event, but we have limited knowledge of how this occurs. Hemolysis is an indicator of membrane interaction, but the hydrolysis of membrane phospholipids can occur without hemolysis. Why are some enzymes active towards liposomes but inactive towards erythrocytes? Why are mixtures of phosphatidylcholine hydrolyzing enzymes and sphingomyelinases hemolytic when the individual enzymes are not? Clearly, substrate specificity is one of the factors that determines the activity of enzymes, but how is this specificity achieved at a molecular level? In spite of research over the past 10 years, we do not fully understand how phospholipases exert their many effects on eukaryotic cells. What role do eukaryotic enzymes play in these processes? How do different enzymes exert different effects on different cell types?

The information that will be derived from these studies over the next decade is of more than philosophical significance. It will allow a variety of new approaches to the control of diseases. In some cases, vaccines will be devised and enzyme inhibitors could be used to treat bacterial diseases. These opportunities provide exciting new challenges to investigators in a rapidly expanding field.

## REFERENCES


Alape-Girón, A., Flores-Díaz, M., Guillouard, I., Naylor, C.E., Titball, R.W., Rucavado, A., Lomonte, B., Basak, A.K., Gutiérrez, J.M., Cole, S.T. and Thelestam, M. (2000). Identification of residues critical for toxicity in *Clostridium perfringens* phospholipase C, the key toxin in gas gangrene. *Eur. J. Biochem*. **267,** 5191–5197.

Angelakopoulos, H., Loock, K., Sisul, D.M., Jensen, E.R., Miller, J.F. and Hohmann, E.L. (2002). Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA/plcB* in adult volunteers: a dose escalation study of oral inoculation. *Infect. Immun*. **70,** 3592–3601.

Antikainen, N.M., Hergenrother, P.J., Harris, M.M., Corbett, W. and Martin, S.F. (2003) Altering substrate specificity of phosphatidylcholine-preferring phospholipase C of *Bacillus cereus* by random mutagenesis of the headgroup binding site. *Biochemistry* **42,** 1603–1610.

Aragon, V., Rossier, O. and Cianciotto, N.P. (2002). *Legionella pneumophila* genes that encode lipase and phospholipase C activities. *Microbiology*, **148,** 2223–2231.

Asmuth, D.M., Olson, R.D., Hackett, S.P., Bryant, A.E., Tweten, R.K., Tso, J.Y., Zollerman, T. and Stevens. D.L. (1995). Effects of *Clostridium perfringens* recombinant and crude phospholipase C and α-toxin on rabbit hemodynamic parameters. *J. Infect. Dis*. **172,** 1317–1323.

Awad, M.M., Bryant, A.E., Stevens, D.L. and Rood, J.I. (1995). Virulence studies on chromosomal α-toxin and θ-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of α-toxin in *Clostridium perfringens*-mediated gas gangrene. *Mol. Microbiol*. **15,** 191–202.

Baine, W.B. (1988). A phospholipase C from the Dallas 1E strain of *Legionella pneumophila* serogroup 5: purification and characterization of conditions for optimal activity with an artificial substrate. *J. Gen. Microbiol*. **134,** 489–498.

Barker, A.P., Vasil, A.I., Filloux, A., Ball, G., Wilderman, P.J. and Vasil, M.L. (2004). A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. *Mol. Microbiol*. **53,** 1089–1098.

Beecher, D.J. and Wong, A.C. (2000). Cooperative, synergistic, and antagonistic hemolytic interactions between hemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from *Bacillus cereus. Microbiology* **146,** 3033–3039.

Bernheimer, A.W., Avigad, L.S. and Kim, K.S. (1974). Staphylococcal sphingomyelinase (β-hemolysin). *Ann. New York. Acad. Sci*. **236,** 292–305.

Blow, D. (1991). Lipases reach the surface. *Nature* **351,** 444–445.

Boethius, J., Rydqvist, B., Möllby, R. and Wadström, T. (1973). Effect of a highly purified phospholipase C on some electrophysiological properties of the frog muscle fiber membrane. *Life Sciences* **13,** 171–176.

Boyd, N.A., Thomson, R.O. and Walker, P.D. (1972). The prevention of experimental *Clostridium novyi* and *Cl.perfringens* gas gangrene in high-velocity missile wounds by active immunisation. *J. Med. Microbiol*. **5,** 467–472.

Bramley, A.J., Patel, A.H., O'Reilly, M. Foster, R. and Foster, T.J. (1989). Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun*. **57,** 2489–2494.

Bruznik, K.S., Morocho, A.M., Jhon, D.-Y., Rhee, S.G. and Tsai, M.-D. (1992) Phospholipids chiral at phosphorous. Stereochemical mechanism for the formation of inositol 1-phosphate catalyzed by phosphatidylinositol-specific phospholipase C. *Biochemistry* **31,** 5183–5193.

Bryant, A.E., Chen, R.Y.Z., Nagata, Y., Wang, Y., Lee, C.H., Finegold, S., Guth, P.H. and Stevens, D.L. (2000). Clostridial gas gangrene I: cellular and molecular mechanisms of microvascular dysfunction induced by exotoxins of *C. perfringens*. *J. Infect. Dis*. **182,** 799–807.

Bryant, A.E., Bayer, C.R., Hayes-Schroer, S.M. and Stevens, D.L. (2003). Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. *J. Infect. Dis*. **187,** 408–417.

Bunting, M., Lorant, D.E., Bryant, A.E., Zimmerman, G.A., McIntyre, T.M., Stevens, D.L. and Prescott, S.M. (1997). Alpha-toxin from *Clostridium perfringens* induces proinflammatory changes in endothelial cells. *J. Clin. Invest*. **100,** 565–574.

Clark, G.C., Briggs, D.C., Karasawa, T., Wang, X., Cole, A.R., Maegawa, T., Jayasekera, P.N., Naylor, C.E., Miller, J., Moss, D.S., Nakamura, S., Basak, A.K. and Titball, R.W. (2003). *Clostridium absonum* alpha-toxin: new insights into clostridial phospholipase C substrate binding and specificity. *J. Mol. Biol*. **333,** 759–769.

De Silva, N.S. and Quinn, P.A. (1987). Rapid screening for phospholipase C activity in mycoplasmas. *J. Clin. Microbiol*. **25,** 729–731.

Dorrell, N., Martino, M.C. and Stabler, R.A. (1999). Characterization of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa. *Gastroenterology* **117,** 1098–1104.

Eaton, J.T., Naylor, C.E., Howells, A.M., Moss, D.S., Titball, R.W. and Basak, A.K. (2002). Crystal structure of the *C. perfringens* alpha-toxin with the active site closed by a flexible loop region. *J. Mol. Biol*. **319,** 275–81.

Edwards, J.L., Entz, D.D. and Apicella, M.A. (2003) Gonococcal phospholipase D modulates the expression and function of complement receptor 3 in primary cervical epithelial cells. *Infect. Immun*. **71,** 6381–6391.

El-Sayed, M.Y., DeBose, C.D., Coury, L.A. and Roberts, M.F. (1985). Sensitivity of phospholipase C (*Bacillus cereus*) activity to phosphatidylcholine structural modifications. *Biochim. Biophys. Acta*. **837,** 325–335.

Exton, J.H. (1990). Signaling through phosphatidylcholine breakdown. *J. Biol. Chem*. **265,** 1–4.

Farn, J.L., Strugnell, R.A., Hoyne, P.A., Michalski, W.P. and Tennent, J.M. (2001) Molecular characterization of a secreted enzyme with phospholipase B activity from *Moraxella bovis*. *J. Bacteriol*. **183,** 6717–6720.

Fiore, A.E., Michalski, J.M., Russell, R.G., Sears, C.L. and Kaper, J.B. (1997). Cloning, characterization, and chromosomal mapping of a phospholipase (lecithinase) produced by *Vibrio cholerae*. *Infect. Immun*. **65,** 3112–3117.

Firth, J.D., Putnins, E.E., Larjava, H and Uitto, V.-J. (1997). Bacterial phospholipase C up-regulates matrix metalloproteinase expression by cultured epithelial cells. *Infect. Immun*. **65,** 4931–4936.

Flieger, A., Neumeister, B. and Cianciotto, N.P. (2002) Characterization of the gene encoding the major secreted lysophospholipase A of *Legionella pneumophila* and its role in detoxification of lysophosphatidylcholine. *Infect. Immun*. **70,** 6094–6106.

Flieger, A., Rydzewski, K., Banerji, S., Broich, M. and Heuner, K. (2004). Cloning and characterization of the gene encoding the major cell-associated phospholipase A of *Legionella pneumophila*, *plaB*, exhibiting hemolytic activity. *Infect. Immun*. **72,** 2648–2658.

Flores-Díaz, M., Alape-Girón, A., Titball, R.W., Moos, M., and Guillouard, I., Cole, S., Howells, A.M.., von Eichel-Streiber, C. and Thelestam, M. (1998). UDP-glucose deficiency causes hypersensitivity to the cytotoxic effect of *Clostridium perfringens* phospholipase C. *J. Biol. Chem*. **273,** 24433–24438.

Fujii, Y., Nomura, S., Oshita, Y. and Sakurai, J. (1986). Excitatory effect of *Clostridium perfringens* alpha toxin on the rat-isolated aorta. *Brit. J. Pharmacol*. **88,** 531–539.

Fujii, Y. and Sakurai, J. (1989). Contraction of the rat-isolated aorta caused by *Clostridium perfringens* alpha-toxin (phospholipase C); evidence for the involvement of arachidonic acid metabolism. *Brit. J. Pharmacol*. **97,** 119–124.

Geoffroy, C., Raveneau, J., Beretti, J.-L., Lechroisey, A., Vaquez-Boland, J.-A., Alouf, J.E. and Berche, P. (1991). Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes*. *Infect. Immun*. **59,** 2382–2388.

Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J. and Goebbel, W. (1989). A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes; nucleotide sequence and genetic linkage. *J. Bacteriol*. **171,** 744–753.

Goldfine, H., Knob, C., Alford, D. and Bentz, J. (1995). Membrane permeabilization by *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C is independent of phospholipid hydrolysis and cooperative with listeriolysin O. *Proc. Natl. Acad. Sci. USA* **92,** 2979–2983.

Granström, M., Erickson, A., Strandvik, B., Wretlind, B., Pavlovskis, O.R., Berka, R. and Vasil, M. (1984). Relationship between antibody response to *Pseudomonas aeruginosa* exoproteins and colonization/infection in patients with cystic fibrosis. *Acta. Paediatr. Scand*. **73,** 772–777.

Gray, G.L., Berka, R.M. and Vasil, M.L. (1982). Phospholipase C regulatory mutation of *Pseudomonas aeruginosa* that results in constitutive synthesis of several phosphate-repressible proteins. *J. Bacteriol*. **150,** 1221–1226.

Guddal, P.H., Johansen, T., Schulstad, K. and Little, C. (1989). Apparent phosphate retrieval system in *Bacillus cereus*. *J. Bacteriol*. **17,** 5702–5706.

Guillouard, I., Garnier, T. and Cole, S.T. (1996). Use of site-directed mutagenesis to probe structure-function relationships of alpha-toxin from *Clostridium perfringens*. *Infect. Immun*. **64,** 2440–2444.

Guillouard, I., Alzari, P.M., Saliou, B. and Cole, S.T. (1997). The carboxy-terminal $C_2$-like domain of the α-toxin from *Clostridium perfringens* mediates calcium-dependent membrane recognition. *Mol. Microbiol*. **26,** 867–876.

Gustafson, C. and Tagesson, C. (1990). Phospholipase C from *Clostridium perfringens* stimulates phospholipase $A_2$ -mediated arachidonic acid release in cultured intestinal epithelial cells (INT 407). *Scand. J. Gastroenterol*. **25,** 363–371.

Hansen, S., Hough, E., Svensson, L.A., Wong, Y.-L. and Martin, S.F. (1993). Crystal structure of phospholipase C from *Bacillus cereus* complexed with a substrate analog. *J. Mol. Biol*. **234,** 179–187.

Haynes, J.A., Tkalcevic, J. and Nisbet, I.T. (1992). Production of an enzymatically inactive analog of phospholipase D from *Corynebacterium pseudotuberculosis*. *Gene* **119,** 119–121.

Heinz, D.W., Essen, L-O. and Williams, R.L. (1998). Structural and mechanistic comparison of prokaryotic and eukaryotic phosphoinositide-specific phospholipases C. *J. Mol. Biol*. **275,** 635–650.

Henner, D.J., Yang, M., Chen, E., Hellmiss, R., Rodriguez, H. and Low, M.G. (1988). Sequence of the *Bacillus thuringiensis* phosphatidylinositol specific phospholipase C. *Nuc. Acids Res*. **16,** 10383.

Hinnebusch, B.J., Cherepanov, P., Du, Y., Rudolph, A., Dixon, J.D., Schwan, T.G. and Forsberg, A. (2000). Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. *Int. J. Med. Microbiol*. **290,** 483–487.

Hinnebusch, B.J., Rudolph, A.E., Cherepanov, P., Dixon, J.E., Schwan, T.G. and Forsberg, A. (2002). Role of Yersinia murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296,** 733–735.

Hodgson, A.L., Krywult, J., Corner, L.A., Rothel, J.S. and Radford, A.J. (1992). Rational attenuation of *Corynebacterium pseudotuberculosis*: potential cheesy gland vaccine and live delivery vehicle. *Infect. Immun*. **60,** 2900–2905.

Holm, B.A., Keicher, L., Liu, M., Sokolowski, J. and Enhorning, G. (1991). Inhibition of pulmonary surfactant function by phospholipases. *J. Appl. Physiol*. **71,** 317–321.

Homma, H., Kobayashi, T, Chiba, N., Karasawa, K., Mizushima, H., Kudo, I., Inoue, K., Ikeda, H., Sekiguchi, M. and Nojima, S. (1994) The DNA sequence encoding *pldA* gene, the structural gene for

detergent-resistant phospholipase A of *E. coli. J. Biochem.* (Tokyo) **96,** 1655–1664.

Hough, E., Hansen, L.K., Birkness, B., Jynge, K., Hansen, S., Hordik, A., Little, C., Dodson, E. and Derewenda, Z. (1989). High-resolution (1.5A) crystal structure of phospholipase C from *Bacillus cereus. Nature* **338,** 357–360.

Ikezawa, H. (1986). The physiological action of bacterial phosphatidylinositol-specific phospholipase C. The release of ectoenzymes and other effects. *J. Toxicol. Toxin Reviews* **5,** 1–24.

Ikezawa, H. (1991). Bacterial PIPLCs-unique properties and usefulness in studies on GPI anchors. *Cell Biol. Int. Reps*. **15,** 1115–1131.

Ikezawa, H., Matsushita, M., Tomita, M. and Taguchi, R. (1986). Effects of metal ions on sphingomyelinase activity of *Bacillus cereus. Arch. Biochem. Biophys*. **249,** 588–595.

Istivan, T.S., Coloe, P.J., Fry, B.N., Ward, P. and Smith, S.C. (2004). Characterization of a hemolytic phospholipase A(2) activity in clinical isolates of *Campylobacter concisus. J. Med. Microbiol*. **53,** 483–493.

Jepson, M., Howells, A., Bullifent, H.L., Bolgiano, B., Crane, D., Miller, J., Holley, J., Jayasekeera, P. and Titball, R.W. (1999). Differences in the carboxy-terminal (putative phospholipid binding) domains of the *Clostridium perfringens* and *Clostridium bifermentans* phospholipases C influence the hemolytic and lethal properties of these enzymes. *Infect. Immun*. **67,** 3297–3301.

Jepson, M., Bullifent, H.L., Crane, D., Flores-Diaz, M., Alape-Giron, A., Jayasekeera, P., Lingard, B. and Titball, R.W. (2001). Tyrosine 331 and phenylalanine 334 in *Clostridium perfringens* alpha-toxin are essential for cytotoxic activity. *FEBS Letts*. **495,** 172–177.

Johansen, K.A., Gill, R.E and Vasil, M.L. (1996). Biochemical and molecular analysis of phospholipase C and phospholipase D activity in Mycobacteria. *Infect. Immun*. **64,** 3259–3266.

Johansen, T., Holm, T., Guddal, P.H., Sletten, K., Haugli, F.B. and Little, C. (1988). Cloning and sequencing of the gene encoding the phosphatidylcholine-preferring phospholipase C of *Bacillus cereus. Gene* **65,** 293–304.

Kameyama, S., Sato, H. and Murata, R. (1975). The role of α-toxin of *Clostridium perfringens* in experimental gas gangrene in guinea pigs. *Jap. J. Med. Sci. Biol*. **25,** 200.

Karasawa, T., Wang, X., Maegawa, T., Michiwa, Y., Kita, H., Miwa, K. and Nakamura, S. (2003). *Clostridium sordellii* phospholipase C: gene cloning and comparison of enzymatic and biological activities with those of *Clostridium perfringens* and *Clostridium bifermentans* phospholipase C. *Infect. Immun*. **71,** 641–646.

Karlyshev, A.V., Oyston, P.C., Williams, K., Clark, G.C., Titball, R.W., Winzeler, E.A. and Wren, B.W. (2001). Application of high-density, array-based, signature-tagged mutagenesis to discover novel yersinia virulence-associated genes. *Infect. Immun*. **69,** 7810–7819.

Klein, R., Miller, N., Kemp, P and Laser, H. (1975). The activation of phospholipase C from *Clostridium perfringens* by quinine: an absolute requirement for calcium ions. *Chem. Phys. Lipids* **15,** 15–26.

König, B., Vasil, M.L. and König, W. (1997). Role of hemolytic and non-hemolytic phospholipase C from *Pseudomonas aeruginosa* for inflammatory mediator release from human granulocytes. *Int. Arch. Allergy. Immunol*. **112,** 115–124.

Kothary, M.H. and Kreger, A.S. (1985). Purification and characterization of an extracellular cytolysin produced by *Vibrio damsela. Infect. Immun*. **49,** 25–31.

Krug, E.L. and Kent, C. (1984). Phospholipase C from *Clostridium perfringens*: preparation and characterization of homogenous enzyme. *Arch. Biochem. Biophys*. **231,** 400–410.

Kuppe, A., Evans, L.M., McMillen, D.A. and Griffith, O.H. (1989). Phosphatidylinositol-specific phospholipase C of *Bacillus cereus*: cloning, sequencing, and relationship to other phospholipases. *J. Bacteriol*. **171,** 6077–6083.

Kurioka, S. and Matsuda, M. (1976). Phospholipase C assay using ρ-nitrophenylphosphorylcholine and its application to studying the metal and detergent requirement of the enzyme. *Anal. Biochem*. **75,** 281–289.

Langton, S.R., and Cesareo, S.D. ( 1992) *Helicobacter pylori* associated phospholipase $A_2$ activity: a factor in peptic ulcer production. *J. Clin Pathol*. **45,** 221–224.

Leao, S.C., Rocha, C.L., Murillo, L.A., Parra, C.A. and Patarroyo, M.E. (1995). A species-specific nucleotide sequence of *Mycobacterium tuberculosis* encodes a protein that exhibits hemolytic activity when expressed in *Escherichia coli. Infect. Immun*. **63,** 4301–4306.

Lee, J.H., Ahn, S.H., Kim, S.H., Choi, Y.H., Park, K.J. and Kong, I.S. (2002). Characterization of *Vibrio mimicus* phospholipase A (PhlA) and cytotoxicity on fish cell. *Biochem. Biophys. Res. Commun*. **298,** 269–276.

Luberto, C., Stonehouse, M.J., Collins, E.A., Marchesini, N., El-Bawab, S., Vasil, A.I, Vasil, M.L. and Hannun, Y.A. (2003). Purification, characterization, and identification of a sphingomyelin synthase from *Pseudomonas aeruginosa*. PlcH is a multifunctional enzyme. *J. Biol. Chem*. **278,** 32733–32743.

MacFarlane, M.G. and Knight, B.C.J.G. (1941). The biochemistry of bacterial toxins. I. Lecithinase activity of *Cl. welchii* toxins. *Biochem. J*. **35,** 884–902.

McNamara, P.J., Bradley, G.A. and Songer, J.G. (1994). Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium pseudotuberculosis. Mol. Microbiol*. **12,** 921–930.

McNee, J.W. and Dunn, J.S. (1917). The method of spread of gas gangrene into living muscle. *Br. Med. J*. **1,** 727–729.

Mengaud, J., Barun-Breton, C. and Cossart, P. (1991). Identification of a phosphatidylinositol-specific phospholipase C in *Listeria monocytogenes*: a novel type of virulence factor? *Mol. Microbiol*. **5,** 367–372.

Möllby, R. (1978). Bacterial phospholipases. In: *Bacterial Toxins and Cell Membranes* (eds. J. Jeljaszewicz and T. Wadström), pp. 367–424. Academic Press, London.

Moreau, H., Pieroni, G., Jolivet-Raynaud, C., Alouf, J.E. and Verger, R. (1988). A new kinetic approach for studying phospholipase C (*Clostridium perfringens* α-toxin ) activity on phospholipid monolayers. *Biochem*. **27,** 2319–2323.

Moser, J., Gerstel, B., Meyer, J.E.W., Chakraborty, T., Wehland, J. and Heinz, D.W. (1997). Crystal structure of the phosphatidylinositol-specific phospholipase C from the human pathogen *Listeria monocytogenes. J. Mol. Biol*. **273,** 269–282.

Murakami, M. and Kudo, I. (2004) Secretory phospholipase A2. *Biol. Pharm. Bull*. **27,** 1158–1164.

Nagahama, M., Michiue, K. and Sakurai, J. (1996). Membrane-damaging action of *Clostridium perfringens* alpha-toxin on phospholipid liposomes. *Biochim. Biophys. Acta*. **1280,** 120–126.

Nagahama, M., Mukai, M., Morimitsu, S., Ochi, S. and Sakurai, J. (2002). Role of the C-domain in the biological activities of *Clostridium perfringens* alpha-toxin. *Microbiol. Immunol*. **46,** 647–655.

Naylor, C.E., Eaton, J.T., Howells, A., Justin, N., Moss, D.S., Titball, R.W. and Basak, A.K. (1998). The structure of the key toxin in gas gangrene. *Nature Struct. Biol*. **5,** 738–746.

Naylor, C.E., Jepson, M., Crane, D.T., Titball, R.W., Miller, J., Basak, A.K. and Bolgiano, B. (1999). Characterization of the calcium-binding C-terminal domain of *Clostridium perfringens* alpha-toxin. *J. Mol. Biol*. **294,** 757–770.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258,** 607–614.

Ochi, S., Hashimoto, K., Nagahama, M. and Sakurai, J. (1996). Phospholipid metabolism induced by *Clostridium perfringens* alpha-toxin elicits a hot-cold type of hemolysis in rabbit erythrocytes. *Infect. Immun*. **64,** 3930–3933.

Ochi, S., Miyawaki, T., Matsuda, H., Oda, M., Nagahama, M. and Sakurai, J. (2002). *Clostridium perfringens* alpha-toxin induces rabbit neutrophil adhesion. *Microbiology*. **148,** 237–245.

Ostroff, R.M., Vasil, A.I. and Vasil, M.L. (1990). Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa. J. Bacteriol*. **172,** 5915–5923.

Otnaess, A-B., Little, C., Sletten, K., Wallin, R., Johnsen, S., Flensgrud, R. and Prydz, H. (1977). Some characteristics of phospholipase C from *Bacillus cereus. Eur. J. Biochem*. **79,** 459–468.

Ottlecz, A., Romero, J.J., Hazell, S.L., Graham, D.Y., Lichtenberger, L.M. (1993). Phospholipase activity of *Helicobacter pylori* and its inhibition by bismuth salts. Biochemical and biophysical studies. *Dig. Dis. Sci*. **38,** 2071–2080.

Parkinson, E.K. (1987). Phospholipase C mimics the differential effects of phorbol-12-myristate-13-acetate on the colony formation and cornification of cultured normal and transformed human keratinocytes. *Carcinogenesis* **8,** 857–860.

Pomerantsev, A.P., Kalnin, K.V., Osorio, M. and Leppla, S.H. (2003). Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. *Infect. Immun*. **71,** 6591–6606.

Preuss, I., Kaiser, I. and Gehring, U. (2001). Molecular characterization of a phosphatidylcholine-hydrolyzing phospholipase C. *Eur. J. Biochem*. **268,** 5081–5091.

Projan, S.J., Kornblum, J., Kreiswirth, B., Moghazeh, S.L., Eiser, W. and Novick, R.P. (1989). Nucleotide sequence of the β-hemolysin gene of *Staphylococcus aureus. Nuc. Acids Res*. **17,** 3305.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, J., Tompkins, R.G. and Ausubel, F.M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268,** 1899–1902.

Raynaud, C., Guilhot, C., Rauzier, J., Bordat, Y., Pelicic, V., Manganelli, R., Smith, I., Gicquel, B. and Jackson, M. (2002). Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis. Mol. Microbiol*. **45,** 203–217.

Renesto, P., Dehoux, P., Gouin, E., Touqui, L., Cossart, P. and Raoult, D. (2003). Identification and characterization of a phospholipase D-superfamily gene in rickettsiae. *J. Infect. Dis*. **188,** 1276–1283.

Rosenthal, A.F. and Pousada, M. (1968). Inhibition of phospholipase C by phosphonate analogues of glycerophosphatides. *Biochim. Biophys. Acta*. **164,** 226–237.

Saiman, L., Cacalano, G., Gruenert, D. and Prince, A. (1992). Comparison of adherence of *Pseudomonas aeruginosa* to respiratory epithelial cells from cystic fibrosis patients and healthy subjects. *Infect. Immun*. **60,** 2808–2814.

Sakurai, J., Ochi, S. and Tanaka, H. (1993). Evidence for coupling of *Clostridium perfringens* alpha toxin-induced hemolysis to stimulate phosphatidic acid formation in rabbit erythrocytes. *Infect. Immun*. **61,** 3711–3718.

Samuelsson, B. (1983). Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science* **220,** 568–575.

Schmiel, D.H., Wagar, E., Karamanou, L. Weeks, D and Miller, V.L. (1998). *Infect. Immun*. **66,** 3941–3951.

Schoepe, H., Pache, C., Neubauer, A., Potschka, H., Schlapp, T., Wieler, L.H., Baljer, G. (2001) Naturally occurring *Clostridium perfringens* non-toxic alpha-toxin variant as a potential vaccine candidate against alpha toxin-associated diseases. *Infect. Immun*. **69,** 7194–6.

Segers, R.P.A.M., van der Drift, A., de Njis, A., Corcione, P., van der Zeijst, B.A.M. and Gaastra, W. (1990). Molecular analysis of a sphingomyelinase C gene from *Leptospira interrogans* serovar hardjo. *Infect. Immun*. **58,** 2177–2185.

Shinoda, S., Matsuoaka, H., Tsuchie, T., Miyoshi, S.-I., Yamamoto, S., Taniguchi, H and Miuguchi, Y. (1991). Purification and characterization of a lecithin-dependent hemolysin from *Escherichia coli* transformed by a *Vibrio parahaemolyticus* gene. *J. Gen. Microbiol*. **137,** 2705–2711.

Shortridge, V.D., Lazdunski, A. and Vasil, M.L. (1992). Osmoprotectants and phosphate regulate expression of phospholipase C in *Pseudomonas aeruginosa. Mol. Microbiol*. **6,** 863–871.

Siboo, R., al-Joburi, W., Gornitsky, M. and Chan, E.C. (1989). Synthesis and secretion of phospholipase C by oral spirochetes. *J. Clin. Microbiol*. **27,** 568–570.

Smith, G.A., Marquis, H., Jones, S., Johnston, N.C., Portnoy, D.A. and Goldfine, H. (1995). The two distinct phospholipase C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. *Infect. Immun*. **63,** 4231–4237.

Songer, J.G. (1997). Bacterial phospholipases and their role in virulence. *Trends in Microbiol*. **5,** 156–161.

Soucek, A., Michalec, C and Souckova, A. (1971). Identification and characterization of a new enzyme of the group "phospholipase D" isolated from *Corynebacterium ovis. Biochim. Biophys. Acta*. **227,** 116–128.

Spiegel, S., Foster, D and Kolesnick, R. (1996). Signal transduction through lipid second messengers. *Curr. Opin. Cell. Biol*. **8,** 159–167.

Stonehouse, M.J., Cota-Gomez, A., Parker, S.K., Martin, W.E., Hankin, J.A., Murphy, R.C., Chen, W., Lim, K.B., Hackett, M., Vasil, A.I. and Vasil, M.L. (2002). A novel class of microbial phosphocholine-specific phospholipases C. *Mol. Microbiol*. **46,** 661–676.

Tannaes, T., Dekker, N., Bukholm, G., Bijlsma, J.J. and Appelmelk, B.J. (2001). Phase variation in the *Helicobacter pylori* phospholipase A gene and its role in acid adaptation. *Infect. Immun*. **69,** 7334–7340.

Thornton, J., Howard, S.P. and Buckley, J.T. (1988). Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferases and other lipases. *Biochim. Biophys. Acta*. **959,** 153–159.

Titball, R.W. (1993). Bacterial phospholipases C. *Microbiol. Revs*. **57,** 347–366.

Titball, R.W., Fearn, A.M. and Williamson, E.D. (1993). Biochemical and immunological properties of the C-terminal domain of the alpha-toxin of *Clostridium perfringens. FEMS Microbiol. Letts*. **110,** 45–50.

Titball, R.W., Hunter, S.E.C., Martin, K.L., Morris, B.C., Shuttleworth, A.D., Rubidge, T., Anderson, D.W. and Kelly, D.C. (1989). Molecular cloning and nucleotide sequence of the alpha-toxin ( phospholipase C ) of *Clostridium perfringens. Infect. Immun*. **57,** 367–376.

Titball, R.W., Leslie, D.L., Harvey, S. and Kelly, D.C. (1991). Hemolytic and sphingomyelinase activities of *Clostridium perfringens* alpha-toxin are dependent on a domain homologous to that of an enzyme from the human arachidonic acid pathway. *Infect. Immun*. **59,** 1872–1874.

Truett, A.P. III and King, L.E. Jr. (1993). Sphingomyelinase D: a pathogenic agent produced by bacteria and arthropods. *Adv. Lip. Res*. **26,** 275–291.

van Deenen, L.L.M., Demel, R.A., Guerts van Kessel, W.S.M., Kamp, H.H., Roelofsen, B., Verkleij, A.J., Wirtz, K.W.A. and Zwaal, R.F.A. (1976). Phospholipases and monolayers as tools in studies on membrane structure. In: *The Structural Basis of Membrane Function*

(eds. Y. Hatefi and L. Djavadi-Ohaniance), pp. 21–38. Academic Press, New York and London.

Voulhoux, R., Ball, G., Ize, B., Vasil, M.L., Lazdunski, A., Wu, L.F. and Filloux, A. (2001). Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. *EMBO J*. **20,** 6735–6741.

Waite, M. (1987). *Handbook of Lipid Research. 5. The phospholipases*. Plenum Press, New York and London.

Walker, N., Holley, J., Naylor, C.E., Flores-Diaz, M., Alape-Giron, A., Carter, G., Carr, F.J., Thelestam, M., Keyte, M., Moss, D.S., Basak, A.K., Miller, J. and Titball, R.W. (2000). Identification of residues in the carboxy-terminal domain of *Clostridium perfringens* α-toxin (phospholipase C), which are required for its biological activities. *Arch. Biochem. Biophys*. **384,** 24–30.

Wieland, C.W., Siegmund, B., Senaldi, G., Vasil, M.L., Dinarello, C.A. and Fantuzzi, G. (2002). Pulmonary inflammation induced by *Pseudomonas aeruginosa* lipopolysaccharide, phospholipase C, and exotoxin A: role of interferon regulatory factor 1. *Infect. Immun*. **70,** 1352–1358.

Wilderman, P.J., Vasil, A.I., Johnson, Z. and Vasil, M.L. (2001) Genetic and biochemical analyses of a eukaryotic-like phospholipase D of *Pseudomonas aeruginosa* suggest horizontal acquisition and a role for persistence in a chronic pulmonary infection model. *Mol. Microbiol*. **39,** 291–303.

Williamson, E.D. and Titball, R.W. (1993). A genetically engineered vaccine against the alpha-toxin of *Clostridium perfringens* also protects mice against experimental gas gangrene. *Vaccine* **11,** 1253–1258.

Yamada, A., Tsukagoshi, N., Ukada, S., Sasaki, T., Makino, S., Nakamura, S., Little, C., Tomita, M. and Ikezawa, H. (1988). Nucleotide sequence and expression in *Escherichia coli* of the gene coding for sphingomyelinase of *Bacillus cereus*. *Eur. J. Biochem*. **175,** 213–220.

Zhang, X., Wehbi, H. and Roberts, M.F. (2004) Cross-linking phosphatidylinositol-specific phospholipase C traps two activating phosphatidylcholine molecules on the enzyme. *J. Biol. Chem*. **279,** 20490–20500.

CHAPTER

# 28

# *Bacteroides fragilis* toxins

*Cynthia L. Sears, Augusto A. Franco, and Shaoguang Wu*

## INTRODUCTION

*Bacteroides fragilis* are common colonic commensals comprising about 0.1% of the fecal flora in the majority, if not all, humans. Despite their small numbers in the fecal flora, *B. fragilis* are the leading anaerobes in intraabdominal and bloodstream infections (Polk and Kasper, 1996). Although the extraintestinal virulence of *B. fragilis* has long been ascribed to the capsule of the organism (Onderdonk *et al*., 1977; Tzianabos *et al*., 1993), recent data has begun to highlight both the genetic and molecular diversity of *B. fragilis* with the recognition that the organism expresses at least eight distinct capsular polysaccharides (Franco *et al*., 1999; Krinos *et al*., 2001; Obuch-Woszczatynski *et al*., 2004). The precise role of the unprecedented capsular diversity of *B. fragilis* in microbial survival, colonization, and disease remains to be defined.

In 1984, the landmark experiments of Myers *et al*. (1984) expanded knowledge on the pathogenic potential of *B. fragilis* and presented the first evidence that certain strains of *Bacteriodes fragilis* (termed enterotoxigenic *B. fragilis* or ETBF) were associated with diarrheal illnesses in young lambs. Subsequent studies revealed that culture supernatants of ETBF strains stimulated intestinal secretion in lamb ligated intestinal loops and that the biologically active factor was a heat-labile, about 20 kDa protein toxin, later termed the *B. fragilis* toxin (BFT) (Myers *et al*., 1984; Myers *et al*., 1985). Since these initial observations, ETBF have also been isolated from patients with extraintestinal *B. fragilis* infections and bacteremia (Chung *et al*., 1999; Claros *et al*., 2000; Kato *et al*., 1996; Pantosti *et al*., 1994a), as well as associated with diarrheal disease in livestock, children (over the age of one year), and adults (Kato *et al*., 2000; Zhang *et al*., 1999; Sears *et al*., 1995; Scotto d'Abusco *et al*., 2000; Sears, 2001). Most recently, in preliminary data, ETBF have been associated with active inflammatory bowel disease (Basset *et al*., 2004; Prindiville *et al*., 2000). The goal of this chapter will be to summarize the progress that has occurred over the last 20 years in understanding the genetics and mechanism of action of BFT.

## THE GENES AND PROTEIN STRUCTURE OF THE *BACTEROIDES FRAGILIS* TOXINS

Initially, the *B. fragilis* toxin (*bft*) gene was cloned and sequenced from three ETBF strains: lamb isolate VPI13874 (VPI-*bft* or *bft-1*), piglet isolate 86-5443-2-2 (86-*bft* or *bft-2*), and systemic isolate Korea 419 (Korea-*bft* or *bft-3*) (Chung *et al*., 1999; Franco *et al*., 1997; Kling *et al*., 1997). The terminology *bft-1*, *bft-2*, and *bft-3* recognizes the temporal sequence in which the alleles were identified. Subsequently, a new *bft* subtype was reported from Japan that is identical to *bft-3* (in amino acid sequence), making this the preferred terminology over Korea-*bft* (Kato *et al*., 2000). Available evidence indicates that ETBF strains and all three *bft* alleles are globally distributed. However, only limited investigations have characterized the epidemiology of the specific *bft* alleles of ETBF. For example, using a collection of 45 intestinal or extraintestinal ETBF strains isolated in the United States, the *bft-1* and *bft-2* alleles were identified in 58% and 42% of strains, respectively (Franco *et al*., 1997), but none was positive for the *bft-3* allele (Chung *et al*., 1999). In contrast, among 34 ETBF strains isolated in Seoul, Korea, 41%, 22%, and 32% contained

*bft-1*, *bft-2*, and *bft-3*, respectively (Chung *et al*., 1999), whereas, among 143 ETBF isolates in Japan, 63%, 24%, and 13% contained *bft-1*, *bft-2*, and *bft-3*, respectively (Kato *et al*., 2000). However, the *bft-3* allele is not restricted to East Asia, as it has been reported in ETBF strains from Europe (d'Abusco *et al*., 2000). Overall, available reports suggest that the *bft-1* allele is most common among human ETBF strains evaluated to date.

Although each of the three *bft* alleles identified and sequenced to date are distinct, all *bft* genes identified are chromosomal, have a guanosine plus cytosine content of 39%, and are predicted to encode a 397-residue holotoxin with a calculated molecular weight of approximately 44.5 kDa. These data, combined with identification of the *N*-terminal amino acid sequence of purified BFT-1 and BFT-2, suggest that ETBF strains synthesize BFT as a pre-proprotein toxin that is then processed by ETBF prior to secretion of the mature biologically active 20 kDa toxin (Figure 28.1) (Franco *et al*., 1997; Kling *et al*., 1997; Van Tassell *et al*., 1992; Wu *et al*., 2002). Consistent with this prediction, approximately 44 kDa and 20 kDa proteins are detected with anti-BFT antisera in lysates of ETBF strains (Franco *et al*., 1999; Franco *et al*., 2005). Fragilysin has been proposed as an alternative name for BFT (Obiso, Jr. *et al*., 1997a), although there is no evidence that BFT induces cell lysis (see Biologic and Physiologic Activities of BFTs, below).

The initial 18 amino acids of the predicted BFT holotoxin encode a signal peptide (preprotein domain) with a pattern typical of lipoprotein signal peptides, although there is no experimental evidence indicating that BFT is a lipoprotein (Franco *et al*., 1997). The proprotein toxin domain is predicted to be 211 residues in length. By analogy with data reported on the function of the proprotein region of the *Pseudomonas aeruginosa* elastase (McIver *et al*., 1995), the BFT proprotein region is hypothesized to be instrumental in proper protein folding and secretion of biologically active BFT (Franco *et al*., 1997). Consistent with these data, cloning and expression of the mature toxin domain without the proprotein domain in *Escherichia coli* yields a biologically inactive protein (Franco and Sears, unpublished data). The high degree of homology of the pre- and proprotein domains of all BFTs (see below and Figure 28.2) is consistent with the interpretation of conserved functions for these protein domains. Cleavage at the Arg-Ala site at the carboxy-terminus of the proprotein domain releases the mature toxin (Franco *et al*., 1997; Van Tassell *et al*., 1992; Wu *et al*., 2002).

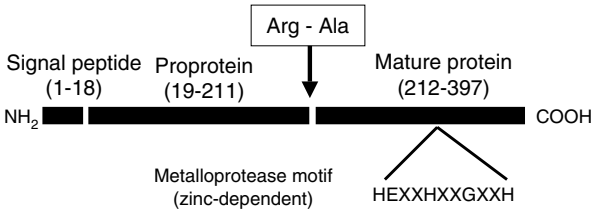



**FIGURE 28.1** Schematic of the protein structure of BFT. The amino acid sequences of BFT-1, BFT-2, and BFT-3 predict that each protein is a pre-proprotein toxin containing signal peptide, proprotein, and mature toxin domains. Intracellular cleavage at the Arg-Ala site by a, as yet unidentified, *B. fragilis* protease yields the mature protein toxin that is secreted by the organism. The mature protein domain of each protein contains a HEXXHXXGXXH motif, suggesting that BFTs are zinc-dependent metalloprotease toxins. Zinc-dependent proteolytic activity has been confirmed for BFT-1 and BFT-2.

The predicted mature toxin domain of each *bft* allele contains an extended zinc-binding metalloprotease motif, HEXXHXXGXXH, and a perfectly superimposable methionine residue close to the metalloprotease motif. This structural constellation predicts that BFTs are members of the metzincin superfamily of zinc-dependent metalloprotease enzymes (Moncrief *et al*., 1995; Obiso, Jr. *et al*., 1997b). BFTs possess the methionine residue 7 amino acids downstream of the zinc-binding metalloprotease motif, typical of the matrix metalloprotease subfamily (matrixins) (Moncrief *et al*., 1995; Obiso, Jr. *et al*., 1997b). Interestingly, limited homology exists between eukaryotic matrix metalloproteases and BFT, leading to the proposal that BFT may be the ancestral origin of these host tissue enzymes (Massova *et al*., 1998). Consistent with the structural predictions, BFT has been shown to be a protease *in vitro* (Moncrief *et al*., 1995) and *in vivo* on cells (Wu *et al*., 1998) and to contain 1 g-atom of $Zn^{2+}$ per toxin molecule (Moncrief *et al*., 1995). In addition, the biological activity of BFT is reduced by approximately 90% by zinc chelation of the purified protein (Koshy *et al*., 1996; Moncrief *et al*., 1995; Obiso, Jr. *et al*., 1995). Experimental proof of the importance of the metalloprotease motif to the biologic activity of BFT is provided by structure:function analyses in which point mutations to modify each of the conserved amino acids of the extended metalloprotease motif, as well as the conserved downstream methionine, reduce or eliminate the biologic activities of BFT (Franco *et al*., 2005). The reported *in vitro* substrates of BFT include G (monomeric)-actin, gelatin, azocoll, tropomyosin, collagen IV, human complement C3, and fibrinogen, although no biologic significance of these substrates has been identified to the cellular or intestinal mechanism of action of BFT (Moncrief *et al*., 1995; Obiso, Jr. *et al*., 1997b; Saidi *et al*., 1997). The only reported cellular (*in vivo*) substrate reported for BFT is E-cadherin (Wu *et al*., 1998). Although BFT has been reported to be autoproteolytic (Moncrief *et al*., 1995; Van Tassell *et al*., 1994a), mutations that inactivate the BFT metallopro-

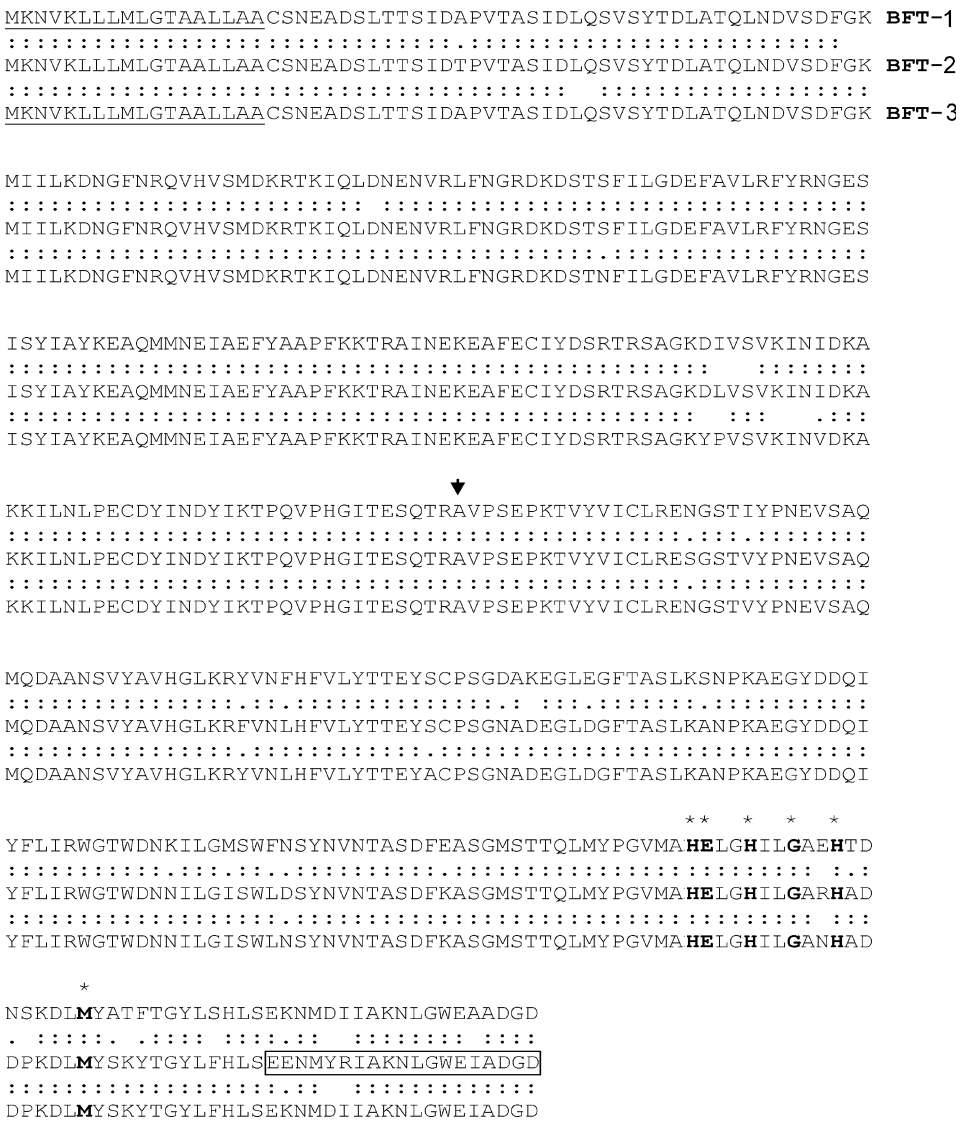



**FIGURE 28.2** Comparison of the amino acid sequences of BFT-1, BFT-2, and BFT-3. BFT-1 and BFT-2 are 95% similar and 92% identical in amino acid sequence. Of note, only two amino acid changes between the two proteins are present in the pro-protein domain, whereas 25 amino acid differences are clustered in the mature toxin domain. BFT-3 is 93% and 96% identical to BFT-1 and BFT-2, respectively. Underlined residues indicate the signal peptide domain. Arrow indicates the Arg-Ala cleavage site yielding release of the mature toxin. * and bold residues indicate the amino acids forming the $Zn^{2+}$-binding metalloprotease motif. Boxed amino acids indicate the predicted amphipathic domain specific to BFT-2.

tease motif do not alter the intracellular processing and secretion of BFT by *B. fragilis*, suggesting that other *B. fragilis* proteases are responsible for the intracellular processing of the toxin (Franco *et al.*, 2005). Protein modeling predicts the presence in BFT-2, but not BFT-1 or BFT-3, of a carboxy-terminus amphipathic domain (Franco *et al.*, 1997). Although this result suggests that the BFT-2 protein may oligomerize and insert into cell membranes, there is no experimental evidence that this occurs as yet.

A direct comparison of the predicted amino acid sequences of BFT-1 and BFT-2 reveals that the predicted proteins are 95% similar and 92% identical (Figure 28.2) (Franco *et al.*, 1997). BFT-3 is more closely related to BFT-2, being 93% and 96% identical with BFT-1 and BFT-2, respectively (Chung *et al.*, 1999). However, the degree of identity varies with the predicted protein domains of the toxins. Namely, comparison of the pre-proprotein domains indicates that these regions are highly conserved with only two to five amino acid changes between BFT-1, BFT-2, and BFT-3 (Chung *et al.*, 1999). In contrast, up to 25 amino acid changes are predicted in the mature toxin protein domain, with the greatest difference occurring between BFT-1 and BFT-2 (Chung *et al.*, 1999; Franco *et al.*, 1997). Detailed structure:function analyses to investigate the biologic importance, if any, of the amino acid heterogeneity in the mature toxin domain have not yet been performed.

Consistent with the protein diversity predicted by the gene sequence, BFT-1 and BFT-2 purify with distinct biochemical profiles and migrate differently when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel

electrophoresis, confirming that the protein products of these *bft* alleles are unique proteins (Wu *et al*., 2002). Consistent with its greater homology to BFT-2, the purification profile of BFT-3 is similar to that of BFT-2 (Chung *et al*., 1999). BFT-2 exhibits modest, but consistently greater specific biologic activity than BFT-1 (Wu *et al*., 2002). The end point titer of biologic activity on HT29/C1 cells (cloned continuous human intestinal epithelial cell line) is 0.37 ± 0.06 ng/ml and 0.13 ± 0.02 ng/ml for BFT-1 and BFT-2 isolated from ETBF strains VPI13874 and 86-5443-2-2, respectively. However, this finding is not known to be important in disease pathogenesis. Both BFT-1 and BFT-2 are trypsin resistant and stable over a wide pH range (i.e., pHs 5-10) (Saidi and Sears, 1996; Van Tassell *et al*., 1992; Wu *et al*., 2002), suggesting mechanisms by which these toxins resist proteolysis in animal and human gut. Details on the properties of BFT-3 are not available as yet.

## MOLECULAR GENETICS OF ETBF

The chromosomes of all ETBF strains possess a unique 6-kb region of DNA not found in non-toxigenic *B. fragilis* strains (Franco *et al*., 1999; Moncrief *et al*., 1998b). This 6-kb DNA region has a guanosine plus cytosine (G + C) content of 35%, which differs from that of the native *B. fragilis* chromosome (about 42%) (Smith *et al*., 1998). Because of the strict association of this 6-kb region with ETBF strains and the G + C content that differs from the native *B.fragilis* chromosome, this DNA region has been named the *B. fragilis* pathogenicity island or islet (BfPAI) (Franco *et al*., 1999; Moncrief *et al*., 1998b). The BfPAI contains the *bft* gene plus about a 700 bp region upstream of *bft* that is required for maximal BFT production by ETBF strains (Franco *et al*., 1999). This 700 bp region contains five putative *B. fragilis* promoter consensus sequences (Bayley *et al*., 2000), although the precise functional promoter sequences have not yet been defined. Besides the *bft* gene, sequence analysis of this 6-kb region from ETBF strains containing *bft-1*, *bft-2*, or *bft-3* revealed one consistent additional open reading frame of 1180 bp with homology to zinc-dependent metalloprotease genes. This gene (termed *mpII* for metalloprotease II) (Moncrief *et al*., 1998b) is predicted to encode a 44.4 kDa protein (termed MPII). MPII has an overall predicted protein structure similar to the BFT proteins (Figure 28.1), namely a pre/signal peptide, proprotein, and mature metalloprotease domains; preliminary data suggest that MPII is also processed to an approximately 20kDa protein (Franco and Sears, unpublished data). However, MPII is only about 56% similar and 28% identical to the BFT proteins, and the lack of homology to the BFT proteins spans all the protein domains. In addition, unlike the BFT proteins, MPII is predicted to contain an ATP/GTP-binding site motif A (P-loop) (Franco *et al*., 1999), suggesting that MPII binds a nucleotide (Saraste *et al*., 1999). To date, no biological activity has been described for MPII, nor has it been completely purified (Franco *et al*., 1999; Moncrief *et al*., 1998a).

Initial studies of the DNA flanking the BfPAI revealed putative mobilization genes, suggesting that the BfPAI may be a mobile genetic element (Franco *et al*., 1999). Further examination of this flanking DNA led to the recognition that this DNA region had a G+C content (47–50%) that differed from the BfPAI (35%) and the *B. fragilis* chromosome (42%), suggesting the flanking DNA was also a distinct genetic element acquired from a different organism (Franco *et al*., 1999). Subsequently, using the Wellcome Trust Sanger Institute *B. fragilis* sequence database (http://www.sanger.ac.uk/Projects/B_fragilis/), the DNA element flanking the BfPAI has been identified to be a member of a new family of putative conjugative transposons (Franco, 2004). These putative conjugative transposons have also been detected in a subset of non-toxigenic *B. fragilis* strains (termed CTn9343 for the *B. fragilis* strain in which the new CTn was first characterized), as well as in ETBF strains (termed CTn86 for the ETBF strain in which the CTn has been partially characterized to date) (Franco, 2004). Distinctive features of these putative CTns are that they lack homology with CTnDOT (a *B. fragilis* CTn) or other described CTns; they lack the *tetQ* gene and tetracycline regulation of chromosomal excision (both features of CTnDOT); and, based on sequence analyses, the mechanism of chromosomal excision and transposition is predicted to differ from other known CTns (Salyers *et al*., 1995; Smith *et al*., 1998). However, neither CTn9343, CTn86, nor the BfPAI has been shown to be transmissible to other *Bacteroides* spp. *in vitro* to date. Intriguingly, using recombinant DNA techniques, *bft* expression appears to be regulated not only by its upstream promoter sequences, but also by one or more genes present on either CTn9343 or CTn86, suggesting that ETBF evolved from commensal, non-toxigenic *B. fragilis* by acquisition of two distinct genetic elements (the BfPAI and CTn86 or CTn9343) (Franco *et al*., 2002). In addition, ETBF strains vary significantly in the HT29/C1 cell biologic activity detected in the culture supernatants of the bacteria (Mundy and Sears, 1996; Myers *et al*., 1987; Van Tassell *et al*., 1994a; Van Tassell *et al*., 1994b; Weikel *et al*., 1992). The mechanisms by which ETBF vary in their secreted biologic activity are unclear, but initial data suggest that ETBF strain differences exist in *bft* gene number, *bft* transcription, and BFT secretion by the organisms (d'Abusco

*et al*., 2000; Wu *et al*., 2002) (unpublished data, Franco and Sears).

## BIOLOGIC AND PHYSIOLOGIC ACTIVITIES OF THE *BACTEROIDES FRAGILIS* TOXINS

Only the activities of BFT-1 and BFT-2 have been examined in any detail in animal models, cultured cells, and monolayers of polarized epithelial cells. Direct "head-to-head" comparisons of these proteins in different experimental models are not available. However, to date, all identified biologic and physiologic activities for BFT-1 and BFT-2 have been very similar, if not identical, except for the modestly higher biologic activity BFT-2 exhibits when tested on the cloned human colonic epithelial cell line, HT29/C1 (Wu *et al*., 2002). However, it is not clear that the observed difference in specific activity of these two proteins is important to the pathogenesis of ETBF disease. Thus, in this section, the biologic and physiologic activities of these proteins will be described without distinguishing between the two isotypes of BFT.

### *In vivo* studies

The activity of purified BFT has been demonstrated in ligated intestinal segments or loops of rats, rabbits, and lambs (Obiso, Jr. *et al*., 1995). These experiments revealed that: (a) histological changes precede the detection of intestinal secretion after treatment with BFT; and (b) BFT stimulates dose-dependent secretion in both ileal and colonic loops in all species examined, although there were species-specific differences in BFT potency in ileal vs. colonic loops (Obiso, Jr. *et al*., 1995). Consistent with these results, previous data in which ETBF organisms were fed to animals suggested that the primary sites of pathology in ETBF intestinal disease were the distal ileum and colon (Collins *et al*., 1989; Duimstra *et al*., 1991). Fluid secreted into the intestinal lumen after treatment with BFT revealed accumulations of sodium and chloride, as well as albumen and protein (Obiso, Jr. *et al*., 1995). At a higher dose of BFT, mildly hemorrhagic fluid and patchy mucosal wall hemorrhage were observed. Consistent with the hypothesis that BFT is an important virulence factor for ETBF *in vivo*, *bft* expression as well as detection of BFT and its biologic activity in feces of animals and humans has been identified (Myers and Weikel, 1992; Pantosti *et al*., 1994b; Pantosti *et al*., 1997; unpublished data, Wu and Sears).

Histological examination of ileal and colonic tissue treated with BFT in all species examined revealed villus blunting, crypt elongation, and neutrophil infiltration consistent with earlier reports of the histopathology of ETBF disease or intestinal tissue exposed to BFT-containing sterile culture filtrates (Obiso, Jr. *et al*., 1995; Sears *et al*., 1995). The inflammatory infiltrate is not always strictly neutrophils, but may be a mixed cell population, including plasma cells and lymphocytes. Extensive detachment and rounding of surface epithelial cells resulting in necrosis of villus tips were also observed. The ileal and colonic tissues of rats were the most severely damaged by BFT. The secretory and histologic effects of purified BFT were partially inhibited by zinc chelation of the toxin (Obiso, Jr. *et al*., 1995). Given that BFT is not a lethal toxin when epithelial cells are treated *in vitro* (Chambers *et al*., 1997; Donelli *et al*., 1996; Koshy *et al*., 1996; Obiso, Jr. *et al*., 1997a; Wells *et al*., 1996), the mechanism(s) leading to tissue damage and hemorrhage are unknown, but one possibility is that the inflammatory response contributes to the tissue damage. This inflammatory response is, at least in part, likely stimulated by BFT (Kim *et al*., 2001; Sanfilippo *et al*., 2000; Wu *et al*., 2004).

### *In vitro* studies of HT29/C1 cells

Early experiments attempting to define the biologic activity of BFT on tissue culture cells failed to identify any activity when crude BFT was tested on Chinese hamster ovary (CHO), Y-1 adrenal, and Vero cells (R.B. Sack, personal communication; Sears *et al*., 1995). However, in 1992, BFT was first reported to exhibit biologic activity on continuous human colonic carcinoma cell lines, HT29/C1, Caco-2, and T84 (Weikel *et al*., 1992). Subsequently, all epithelial cell lines examined to date with the capability of forming polarized monolayers *in vitro* (e.g., intestinal [HT29/C1, Caco-2, T84], renal [MDCK], and pulmonary [Calu-3] cell lines) developed morphologic changes in response to BFT (Figure 28.3). Other continuous colonic carcinoma cell lines, such as SW480 and HCT116, are also responsive to BFT (Wu *et al*., 2003; unpublished data, Wu and Sears). Subconfluent, uncloned HT29 or cloned HT29/C1 cell lines have been studied in the greatest detail and, in particular, cloned HT29/C1 cells have been shown to be exquisitely sensitive to BFT with a half-maximal effective toxin concentration of approximately 12.5 pM and as little as 0.5 pM altering cell morphology (Saidi and Sears, 1996). Using the HT29/C1 cell line, the key morphologic observation is that all BFTs cause rounding of the cells and disruption of cell-to-cell contacts, leading to dispersion of the cells from their normally tight cluster morphology (Figure 28.3a) (Chung *et al*., 1999; Mundy and Sears, 1996; Weikel *et al*., 1992). These changes in the light microscopic appearance of the cells

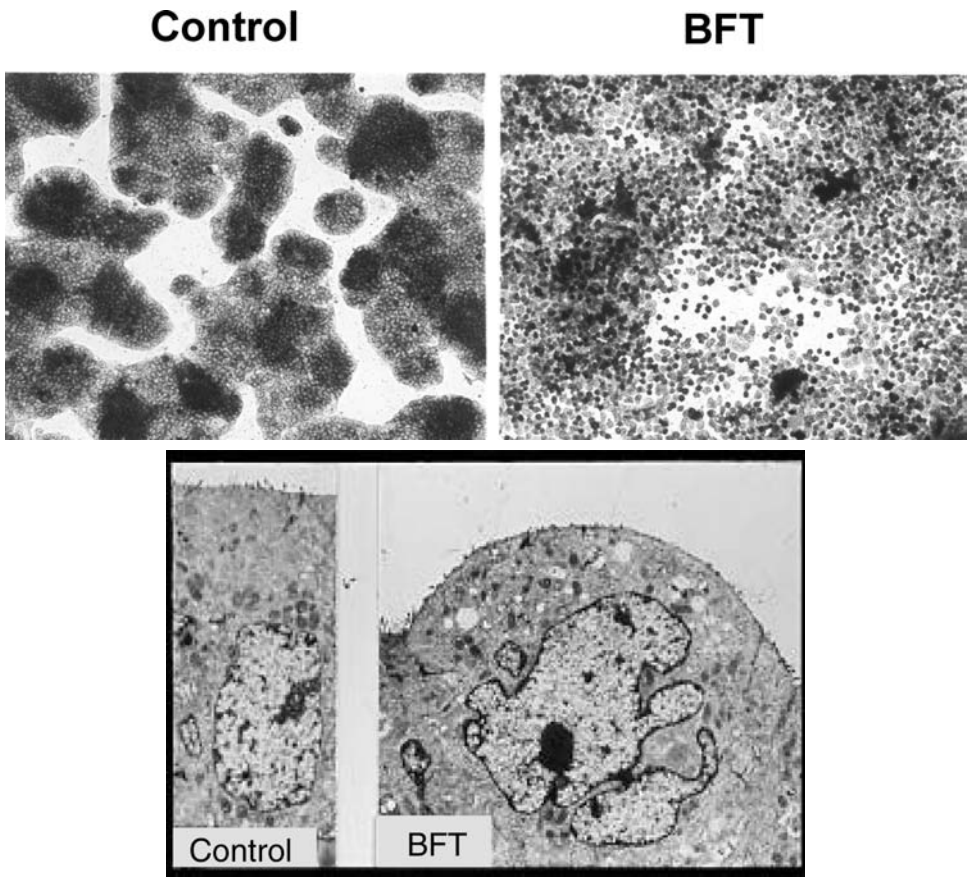



**FIGURE 28.3** Effect of BFT on intestinal epithelial cells *in vitro*. Light microscopic appearance of HT29/C1 cells after treatment with BFT (5 nM, 3 h). BFT-treated subconfluent HT29/C1 cells are rounded with loss of the tight cluster morphology when compared with untreated control cells (original magnification × 100). Transmission electron micrograph of a BFT-treated (5 nM, 2 h) polarized T84 cell. The BFT-treated cell reveals a dramatic increase in cell size and development of a domed apical membrane with loss of intact microvilli (original magnification × 3000). (Reproduced with permission from Chambers *et al*., 1997.)

are accompanied by time- and concentration-dependent changes in the F (filamentous)-actin structure of the cells, although the total F-actin content of the cells is unaltered (Donelli *et al*., 1996; Koshy *et al*., 1996; Saidi and Sears, 1996). Decreased stress fibers, cell surface blebs, and floccular F-actin staining with peripheral F-actin condensation are observed (Donelli *et al*., 1996; Koshy *et al*., 1996). The cellular morphologic changes stimulated by BFT require cellular ATP, indicating, not unexpectedly, that the BFT-stimulated changes in cell shape are energy-dependent events (Wu *et al*., 1998). However, further mechanistic details regarding how BFT modifies cell morphology and cellular F-actin structure are not yet known.

In studies of subconfluent HT29/C1 cells, BFT alters the morphology of HT29/C1 cells in a time-, temperature- and concentration-dependent manner (Saidi and Sears, 1996; Wu *et al*., 1998). BFT is inactive at 4°C, partially active at 20°C, and fully active at 37°C (Moncrief *et al*., 1995; Saidi and Sears, 1996). At 37°C, the onset of action of BFT is rapidly irreversible to washing. After treatment of HT29/C1 cells with 0.25 nM BFT for only 7 min or 2.5 nM for 2 min (followed by washing to remove the toxin), cells treated with BFT are destined to change shape. HT29/C1 cells will begin to develop altered morphology as early as about 10 min after BFT treatment, with 100% of subconfluent HT29/C1 cells exhibiting rounding by 30–60 min (Saidi and Sears, 1996; Wu *et al*., 1998). Changes in cell morphology become even more dramatic over the next 3 to 6 hours (Saidi and Sears, 1996; Weikel *et al*., 1992).

Despite the dramatic changes in morphology, HT29/C1 cells regain a normal appearance by light microscopy by 2–3 days after toxin treatment, indicating the biologic activity of BFT on HT29/C1 cells is reversible (Saidi and Sears, 1996; Weikel *et al*., 1992; Wu *et al*., 1998). It is unknown if this is due, for example, to loss of BFT biologic activity (BFT is a heat-labile protein), to proteolysis of BFT by host cells, or to other mechanisms of cell recovery. Further, most available evidence indicates that BFT is a non-lethal and non-cytotoxic protein. Namely, protein synthesis is not diminished in BFT-treated cells and, in fact, is stimulated beginning approximately 5 h after BFT treatment (Chambers *et al*., 1997; Koshy *et al*., 1996); LDH release from BFT-treated cells is not observed (Chambers *et al*., 1997; Koshy *et al*., 1996); BFT-treated cells exclude trypan blue and propidium iodide (Obiso *et al*., 1997a; Wells *et al*., 1996); DNA synthesis continues normally (Donelli *et al*., 1996); and no $^{51}Cr$ release from BFT-treated cells is observed (Obiso *et al*., 1997a). However, one report suggests that BFT induces cell death with

features of apoptosis by electron microscopy in cells released from plastic by BFT (Sanfilippo *et al*., 2000). Inhibitors of microtubules, and endosomal or Golgi trafficking do not alter BFT's activity (Donelli *et al*., 1996; Obiso, Jr. *et al*., 1997a; Saidi and Sears, 1996).

## HT29/C1 cells: molecular mechanism of action of BFT

Because BFT does not appear to enter cells (Donelli *et al*., 1996; Obiso, Jr. *et al*., 1997a; Saidi and Sears, 1996), and its biologic activity on intestinal epithelial cells is strikingly polar (Chambers *et al*., 1997; Obiso *et al*., 1997a), Wu *et al*. (1998) hypothesized that the cellular substrate for BFT was a cell surface protein present on the basolateral membrane of intestinal epithelial cells. Experiments to address this hypothesis revealed that BFT does not cleave the zonula occludens proteins, occludin or claudins (1 or 2), or the basal membrane protein, $\beta_1$-integrin, although these proteins have extracellular domains presumably accessible to BFT's proteolytic activity (Wu *et al*., 1998; unpublished data, Wu and Sears). Similarly, consistent with its proposed site of action at the cell surface, no quantitative changes in the intracellular proteins, $\beta$-catenin, $\alpha$-catenin, zonula occludens protein-1 (ZO-1), or actin, are identified by Western blot analysis during the first hour of BFT treatment of intestinal epithelial cells (Saidi *et al*., 1997; Wu *et al*., 1998). However, although these proteins are not BFT substrates, analysis of the cellular distribution of ZO-1, $\beta$-catenin, or occludin, for example, by confocal microscopy reveals redistribution of these proteins in the cells by one hour after treatment with BFT (Obiso *et al*., 1997a; Wu *et al*., 1998).

In contrast, the zonula adherens protein, E-cadherin (a 120 kDa transmembrane protein), is rapidly cleaved by BFT, as demonstrated by Western blot and confocal immunofluorescence analysis (Wu *et al*., 1998). This activity of BFT has been examined in greatest detail using HT29/C1 cells, although E-cadherin is also cleaved on polarized T84 and MDCK cells. Presentation of E-cadherin on an intact, living cell is required to detect E-cadherin cleavage stimulated by BFT, as *in vitro* cleavage of E-cadherin cannot be demonstrated in approaches tested to date (Wu *et al*., 1998). E-cadherin cleavage stimulated by BFT is time- and concentration-dependent. Onset of E-cadherin cleavage is detectable by 1 minute after treatment of intestinal epithelial cells (HT29/C1) with 5 nM BFT and yields 33 kDa and 28 kDa cell-associated E-cadherin fragments. By 1–2 hours after BFT treatment, there is a complete loss of intact cellular E-cadherin. The size of the proteolytic degradation products of E-cadherin combined with data indicating that BFT does not enter cells (Donelli *et al*., 1996; Obiso *et al*., 1997a; Saidi and Sears, 1996) and does not require cellular ATP for the initial cleavage of E-cadherin (Wu *et al*., 1998) suggest that BFT acts as a specific cell-surface protease toxin cleaving E-cadherin in its extracellular domain near the cellular plasma membrane. This finding has recently been confirmed using proteolytically inactive BFT mutants (unpublished data, Wu, Franco, and Sears). However, the exact site of E-cadherin cleavage has not yet been identified. Consistent with these data indicating that BFT cleaves E-cadherin, Wells *et al*. (1996) reported that treatment of HT29 cells with BFT resulted in decreased internalization of *Listeria monocytogenes* (but not other pathogenic enteric bacteria). E-cadherin is one ligand utilized by *L. monocytogenes* to enter epithelial cells (Mengaud *et al*., 1996). In contrast, further degradation of the 33 kDa and 28 kDa cell-associated E-cadherin fragments released by BFT treatment of cells requires cellular ATP, suggesting the recruitment of host cell proteases at this step in the mechanism of action of BFT (Wu *et al*., 1998). Consistent with this hypothesis, recent data suggest that presenilin-1/γ-secretase and proteasomal enzymes contribute sequentially to the degradation of the BFT-released 33 kDa and 28 kDa E-cadherin fragments (unpublished data, Wu and Sears). Cellular recovery after BFT treatment correlates with resynthesis of E-cadherin. However, transcriptional up-regulation of E cadherin synthesis has not been demonstrated (Wu *et al*., 1998).

Besides the critical role of its extracellular domain in intercellular adhesion, E-cadherin is complexed at its intracellular domain to β–catenin, a nuclear signaling protein involved in both normal and dysregulated cellular growth (Nelson and Nusse, 2004). Thus, the recognition that BFT induces proteolysis of E-cadherin led to the hypothesis that BFT may trigger β–catenin cellular signaling and cell proliferation. Indeed, BFT treatment of HT29/C1 cells induces β–catenin nuclear localization within 3 hours followed by induction of *c-myc* (a β–catenin-regulated gene) transcription and translation (Wu *et al*., 2003). By 48 hours after BFT treatment of HT29/C1 cells, cellular proliferation ensues and is stimulated by as little as 0.5 nM BFT (Wu *et al*., 2003). However, β–catenin signaling accounts for only about 30–40% of BFT-induced cellular proliferation, indicating that other, as yet unidentified, mechanisms contribute to cell growth stimulated by BFT (Wu *et al*., 2003). Of note, diminished E-cadherin on tumor cells is associated with enhanced metastatic potential and, consistent with this observation, BFT-treated HT29/C1 cells are highly mobile for up to 96 hours after treatment with BFT (unpublished data, Wu and Sears).

The pathology of ETBF-infected ileum and colon, as well as intestinal tissues treated with BFT, suggested

that BFT induces an inflammatory response (Obiso *et al*., 1995; Sears *et al*., 1995). Recently, BFT has been reported to stimulate secretion of the proinflammatory chemokine, interleukin-8 (IL-8), by human intestinal epithelial cells (HT29, HT29/C1, T84, and Caco-2) (Kim *et al*., 2001; Sanfilippo *et al*., 2000; Wu *et al*., 2004). Induction of IL-8 mRNA expression occurs rapidly and ceases by 6 hours after BFT treatment, whereas IL-8 secretion continues for at least 18 hours (Wu *et al*., 2004). Initial data indicate that multiple cellular signal transduction pathways contribute to BFT-induced IL-8 secretion, including the mitogen-activated protein kinases (MAPKs), p38 and extracellular signal-related kinase (ERK), tyrosine kinases, and Nuclear Factor-κβ (Kim *et al*., 2001; Wu *et al*., 2004). BFT induces an unusual supranuclear localization of Nuclear Factor-κβ that is dependent on activation of one or more tyrosine kinases; and simultaneous activation of MAPKs and Nuclear Factor-κβ appears necessary for induction of IL-8 secretion by BFT (Wu *et al*., 2004). Stimulation of IL-8 secretion *in vivo* is one potential mechanism by which ETBF, via BFT, may result in an intestinal inflammatory response. BFT has also been reported to induce increased expression of other chemokines important for the chemoattraction and activation of neutrophils, epithelial-neutrophil activating protein-78 (ENA-78), and growth-related oncogene-α (GRO-α), as well as transforming growth factor-β (TGF-β), an intestinal repair cytokine. However, the secreted TGF-β stimulated by BFT was largely in a biologically inactive form (Sanfilippo *et al*., 2000).

## *In vitro* studies with polarized epithelial monolayers

When polarized monolayers of epithelial cells (T84, MDCK, HT29, HT29/C1, Caco-2) are tested with BFT *in vitro*, the most striking observations are that: (a) BFT decreases the resistance of epithelial monolayers in a dose- and time-dependent manner; and (b) the activity of the toxin is polar; namely, BFT decreases resistance more rapidly and at lower toxin concentrations when placed on the basolateral membranes of the cell monolayers than when placed on the apical membranes of the monolayers (Chambers *et al*., 1997; Obiso *et al*., 1997a; Sears *et al*., 1995; Wells *et al*., 1996; unpublished data, Wu and Sears). Apical treatment of T84, MDCK, or HT29 monolayers with BFT (5 nM) results in an approximately 50% decrease in monolayer resistance after 3 to 6 hours (Chambers *et al*., 1997; Obiso *et al*., 1997a). In contrast, treatment of the basolateral membranes of T84 monolayers with BFT decreases monolayer resistance by 90% within 2 hours (Chambers *et al*., 1997). In addition, basolateral BFT rapidly increases the short circuit current (Isc; indicative of chloride secretion) in a concentration-dependent manner, a finding not observed with apical BFT (Chambers *et al*., 1997). The increase in Isc stimulated by BFT is self-limited, returning to baseline as monolayer resistance decreases further. T84 monolayers also secrete IL-8 in a polar manner when treated with BFT with predominant release of this chemokine across the basolateral membrane of the monolayers (Kim *et al*., 2001; Wu *et al*., 2004). This result suggests that BFT contributes to induction of the submucosal inflammatory response observed *in vivo* in response to ETBF infection.

Consistent with these physiologic results, apical BFT causes focal changes in the morphology of T84 monolayers, whereas basolateral BFT alters the morphology of all cells in the monolayer with development of a striking domed apical membrane on the cells (Chambers *et al*., 1997). Transmission and scanning electron microscopy reveal that cell swelling occurs with a loss of the microvilli of the apical membrane, resulting in the domed membrane structure (Figure 28.3b). Consistent with these observations, BFT stimulates a protracted increase in HT29/C1 cell volume, although the mechanism of this effect is unknown (Koshy *et al*., 1996). By transmission electron microscopy, the zonula occludens (tight junction) and zonula adherens, electron-dense structures that regulate the "barrier function" of epithelial monolayers, are observed to develop concentration-dependent morphologic changes. Initially, these structures become more dense and compact after BFT treatment, with subsequent complete loss of these structures between some cells. Sites where the zonula occludens and zonula adherens are noted to dissolve are typically associated with a complete loss of the apical membrane microvillus structure. These morphological changes explain the measured decrease in monolayer resistance ("barrier function"). Interestingly, the development of gaping junctions between HT29 cells treated with BFT has been shown to permit increased association and invasion of the basolateral membranes with pathogenic enteric bacteria (Wells *et al*., 1996). Despite the striking changes observed in the zonula occludens and zonula adherens, desmosomes remain intact after BFT treatment *in vivo* and *in vitro* (Chambers *et al*., 1997; Sears *et al*., 1995).

When F-actin is examined by confocal microscopy in T84 monolayers treated with basolateral BFT, the actin of the tight junctional ring and microvilli is nearly absent (Chambers *et al*., 1997). However, increased F-actin is demonstrated at the basal pole of the cells, consistent with the observation in HT29/C1 cells that total F-actin is unchanged in BFT-treated cells (Saidi *et al*., 1997). These results suggest that treatment of epithelial cells with BFT stimulates dissociation and

reassociation of F-actin, albeit in an entirely new cellular location. Interestingly, treatment of human colon *in vitro* with BFT confirms the polar activity of BFT on barrier function (tissue resistance) and the altered cellular F-actin architecture (Riegler *et al*., 1999). Morphologic changes in response to BFT have also been observed in organ cultures of human colonic biopsies, although heterogeneity in the response was observed, suggesting potential variable susceptibility to BFT and possibly ETBF disease in humans (Sanfilippo *et al*., 1998). Last, the ability of BFT to modify mucosal permeability has been proposed as the mechanism whereby BFT acts as a mucosal adjuvant to enhance the systemic antibody response to an intranasal antigen challenge in mice (Vines *et al*., 2000).

## Models of the mechanism of action of BFT and ETBF pathogenesis

Figure 28.4 shows proposed models of the molecular mechanism of action of BFT and the pathogenesis of ETBF-mediated intestinal secretion. Although these

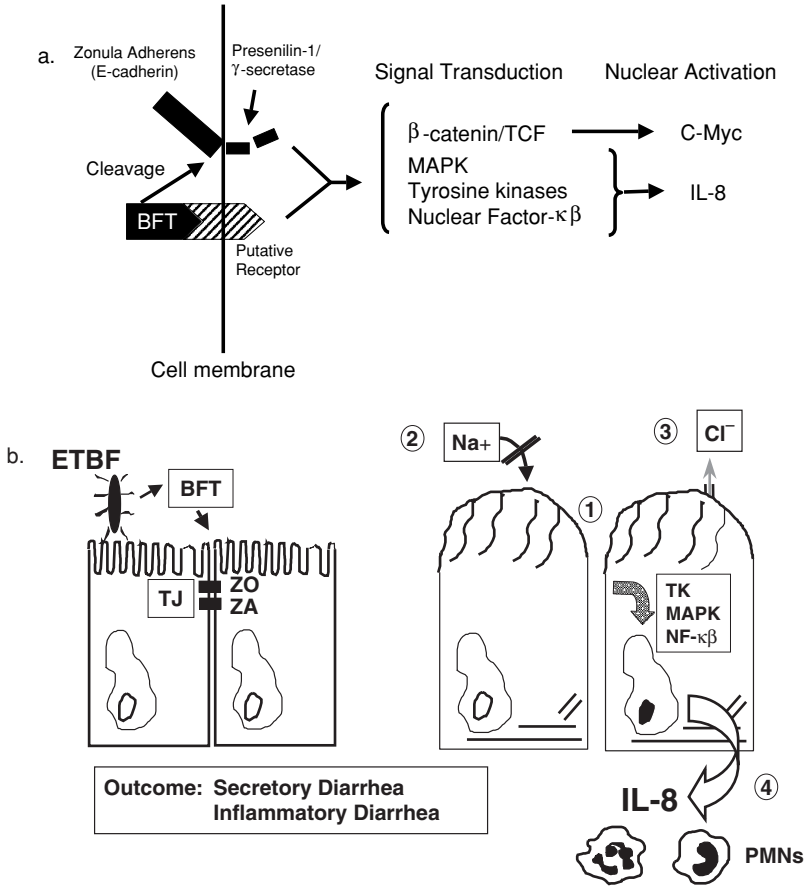



**FIGURE 28.4** Proposed models of BFT and ETBF pathogenesis. (a) Molecular mechanism of action of BFT. BFT binds to a specific cellular receptor (unpublished data, Wu and Sears) and cleaves the extracellular domain of the zonula adherens protein, E-cadherin, in an ATP-independent manner. Modification of the extracellular domain of E-cadherin triggers the ATP-dependent proteolysis of the intracellular domain of E-cadherin, probably by presenilin-1/γ-secretase and proteasomal enzymes. Loss of intact E-cadherin disrupts its linkages with catenin proteins and hence F-actin (not shown). These events may lead to the characteristic disruption of the apical cytoskeleton of polarized epithelial cells (Chambers *et al*., 1997; and Figure 28.3b. BFT also stimulates activation of multiple signal transduction pathways, resulting in nuclear activation and induction of new protein synthesis. Both C-Myc and interleukin-8 (IL-8) have been demonstrated to be induced in BFT-treated HT29/C1 cells (see text). TCF: T Cell Factor; MAPK: mitogen-activated protein kinases. (b) Pathogenesis of ETBF diarrheal disease. ETBF organisms attach to the apical membrane of intestinal epithelial cells and secrete BFT. BFT is proposed to diffuse through the zonula occludens (ZO) to reach the zonula adherens (ZA) where BFT cleaves its target protein E-cadherin. Cleavage of E-cadherin precipitates focal morphological changes and diminished barrier function (1). These events facilitate the delivery of BFT to the basolateral membranes of intestinal epithelial cells where further cleavage of E-cadherin may augment the apical morphological changes initially simulated by BFT. The dramatic changes in the apical morphology of BFT-treated intestinal epithelial cells are hypothesized to alter the function of one or more ion transporters (2,3), resulting in net intestinal secretion. Further, cellular signal transduction stimulated by BFT induces IL-8 synthesis and secretion (along with possibly other pro-inflammatory cytokines) into the submucosa resulting in an inflammatory response (4). 1 to 4 indicate proposed mechanisms by which ETBF, via BFT, stimulate intestinal secretion. TJ: Tight junction; TK: Tyrosine kinases; NF-κβ: Nuclear Factor-κβ; PMNs: polymorphonuclear leukocytes.

models build on the data on the biochemical, biologic, and physiologic sequelae of treatment of intestinal epithelial cells with BFT discussed in this chapter, many of the proposed steps require additional investigation for validation.

## FUTURE CHALLENGES IN ETBF AND BFT RESEARCH

The cleavage of E-cadherin by BFTs represents the first time that E-cadherin has been described as a bacterial toxin substrate. In addition, BFT is the first cytoskeletal-altering toxin predicted to act solely at the cell surface and without covalent enzymic modification of an intracellular substrate. Remodeling of cell-surface proteins by extracellular proteases is a mechanism yielding specific cellular responses and triggering specific cellular signal transduction pathways (Werb, 1997; Werb and Yan, 1998). The data available both on the dramatic cellular responses and cell signaling stimulated by BFT serve to illustrate this principle. Future studies to identify the mechanisms by which BFT induces cellular proliferation, inflammation, and actin cytoskeletal rearrangement are expected to contribute to the understanding of both normal and pathologic cell function. Importantly, the ability of BFT to induce cellular proliferation and inflammation suggests the hypothesis that prolonged mucosal colonization with ETBF may contribute to the pathogenesis of intestinal inflammatory and oncogenic processes. Consistent with this hypothesis, recent studies have suggested an association between ETBF colonization and active inflammatory bowel disease (Basset *et al*., 2004; Prindiville *et al*., 2000).

## ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Award RO1 DK 45496 (to CLS) and A148708 (to AAF).

## REFERENCES

Basset, C., Holton, J., Bazeos, A., Vaira, D. and Bloom, S. (2004). Are Helicobacter species and enterotoxigenic *Bacteroides fragilis* involved in inflammatory bowel disease? *Dig. Dis. Sci*. **49,** 1425–1432.

Bayley, D.P., Rocha, E.R. and Smith, C.J. (2000). Analysis of cepA and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol. Lett*. **193,** 149–154.

Chambers, F.G., Koshy, S.S., Saidi, R.F., Clark, D.P., Moore, R.D. and Sears, C.L. (1997). *Bacteroides fragilis* toxin exhibits polar activity on monolayers of human intestinal epithelial cells (T84 cells) *in vitro*. *Infec. Immun*. **65,** 3561–3570.

Chung, G.T., Franco, A.A., Wu, S., Rhie, G.E., Cheng, R., Oh, H.B. and Sears, C.L. (1999). Identification of a third metalloprotease toxin gene in extraintestinal isolates of *Bacteroides fragilis*. *Infection and Immunity* **67,** 4945–4949.

Claros, M.C., Claros, Z.C., Tang, Y.J., Cohen, S.H., Silva, J., Jr., Goldstein, E.J. and Rodloff, A.C. (2000). Occurrence of *Bacteroides fragilis* enterotoxin gene-carrying strains in Germany and the United States. *J. Clin. Microbiol*. **38,** 1996–1997.

Collins, J.H., Bergeland, M.E., Myers, L.L. and Shoop, D.S. (1989). Exfoliating colitis associated with enterotoxigenic *Bacteroides fragilis* in a piglet. *J. Vet. Diagn. Invest*. **1,** 349–351.

d'Abusco, A.S., Del Grosso, M., Censini, S., Covacci, A. and Pantosti, A. (2000). The alleles of the bft gene are distributed differently among enterotoxigenic *Bacteroides fragilis* strains from human sources and can be present in double copies. *J. Clin. Microbiol*. **38,** 607–612.

Donelli, G., Fabbri, A. and Fiorentini, C. (1996). *Bacteroides fragilis* enterotoxin induces cytoskeletal changes and surface blebbing in HT-29 cells. *Infection and Immunity* **64,** 113–119.

Duimstra, J.R., Myers, L.L., Collins, J.E., Benfield, D.A., Shoop, D.S. and Bradbury, W.C. (1991). Enterovirulence of enterotoxigenic *Bacteroides fragilis* in gnotobiotic pigs. *Vet. Pathol*. **28,** 514–518.

Franco, A.A., Mundy, L.M., Trucksis, M., Wu, S., Kaper, J.B. and Sears, C.L. (1997). Cloning and characterization of the *Bacteroides fragilis* metalloprotease toxin gene. *Infec. Immun*. **65,** 1007–1013.

Franco, A.A., Cheng, R.K., Chung, G.T., Wu, S., Oh, H.B. and Sears, C.L. (1999). Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. *J. Bacteriol*. **181,** 6623–6633.

Franco, A.A., Cheng, R.K., Goodman, A. and Sears, C.L. (2002). Modulation of *bft* expression by the *Bacteroides fragilis* pathogenicity island and its flanking region. *Mol. Micro.* **45,** 1067–1077.

Franco, A.A. (2004). The *Bacteroides fragilis* pathogenicity island is contained in a putative novel conjugative transposon. *J. Bacteriol*. **186,** 6077–6092.

Franco, A. A., Buckwold, S., Shin, J.W., Ascon, M. and Sears, C.L. (2005). Mutation of the zinc-binding metalloprotease motif affects *Bacteroides fragilis* toxin activity without affecting propeptide processing. *Infec. Immun*. In press.

Kato, N., Kato, H., Watanabe, K. and Ueno, K. (1996). Association of enterotoxigenic *Bacteroides fragilis* with bacteremia. *Clin. Infect. Dis*. **23** *Suppl 1*, S83–S86.

Kato, N., Liu, C.X., Kato, H., Watanabe, K., Tanaka, Y., Yamamoto, T., Suzuki, K. and Ueno, K. (2000). A new subtype of the metalloprotease toxin gene and the incidence of the three bft subtypes among *Bacteroides fragilis* isolates in Japan. *FEMS Microbiol. Lett*. **182,** 171–176.

Kim, J.M., Oh, Y.K., Kim, Y.J., Oh, H.B. and Cho, Y.J. (2001). Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to *Bacteroides fragilis* enterotoxin: NF-kappa B plays a major role in the regulation of IL-8 expression. *Clin. Exp. Immunol*. **123,** 421–427.

Kling, J.J., Wright, R.L., Moncrief, J.S. and Wilkens, T.D. (1997). Cloning and characterization of the gene for the metalloprotease enterotoxing of *Bacteroides fragilis*. *FEMS Microbiol Letters* **146,** 279–284.

Koshy, S.S., Montrose, M.H. and Sears, C.L. (1996). Human intestinal epithelial cells swell and demonstrate actin rearrangement in response to the metalloprotease toxin of *Bacteroides fragilis*. *Infec. Immun*. **64,** 5022–5028.

Krinos, C.M., Coyne, M.J., Weinacht, K.G., Tzianabos, A.O., Kasper, D.L. and Comstock, L.E. (2001). Extensive surface diversity of a

commensal microorganism by multiple DNA inversions. *Nature* **414,** 555–558.

Massova, I., Kotra, L.P., Fridman, R. and Mobashery, S. (1998). Matrix metalloproteinases: structures, evolution, and diversification. *FASEB J*. **12,** 1075–1095.

McIver, K.S., Kessler, E., Olson, J.C. and Ohman, D.E. (1995). The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa*. *Mol. Microbiol*. **18,** 877–889.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R. and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. Monocytogenes* into epithelial cells. *Cell* **84,** 923–932.

Moncrief, J.S., Obiso, R., Barroso, L.A., Kling, J.J., Wright, R.L., Van Tassell, R.L., Lyerly, D.M. and Wilkins, T.D. (1995). The enterotoxin of *Bacteroides fragilis* is a metalloprotease. *Infection and Immunity* **63,** 175–181.

Moncrief, J.S., Barroso, L.A. and Wilkins, T.D. (1998a). Purification and Characterization of Recombinant Metalloprotease II from Enterotoxigenic *Bacteroides fragilis*. *98th General Meeting, American Society of Microbiology*, Atlanta, Georgia, B-274.

Moncrief, J.S., Duncan, A.J., Wright, R.L., Barroso, L.A. and Wilkins, T.D. (1998b). Molecular characterization of the fragilysin pathogenicity islet of enterotoxigenic *Bacteroides fragilis*. *Infec. Immun*. **66,** 1735–1739.

Mundy, L.M. and Sears, C.L. (1996). Detection of toxin production by *Bacteroides fragilis*: assay development and screening of extraintestinal clinical isolates. *Clin. Infect. Dis*. **23,** 269–276.

Myers, L.L., Firehammer, B.D., Shoop, D.S. and Border, M.M. (1984). *Bacteroides fragilis*: a possible cause of acute diarrheal disease in newborn lambs. *Infection and Immunity* **44,** 241–244.

Myers, L.L., Shoop, D.S., Firehammer, B.D. and Border, M.M. (1985). Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in calves. *Journal of Infectious Diseases* **152,** 1344–1347.

Myers, L.L., Shoop, D.S., Stackhouse, L.L., Newman, F.S., Flaherty, R.J., Letson, G.W. and Sack, R.B. (1987). Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. *J. Clin. Microbiol*. **25,** 2330–2333.

Myers, L.L. and Weikel, C.S. (1992). Enterotoxin as a virulence factor in *bacteroides fragilis*—associated diarrheal disease. In: *Medical and Environmental Aspects of Anaerobes* pp. 90–100 (eds. B.I. Duerden, J.S. Brazier, S.V. Seddon, W.G. WadePetersfield): Wrightson Biomedical Publishing Ltd., U.K.

Nelson, W.J. and Nusse, R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* **303,** 1483–1487.

Obiso, R.J., Jr., Lyerly, D.M., Van Tassell, R.L. and Wilkins, T.D. (1995). Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage *in vivo*. *Infection and Immunity* **63,** 3820–3826.

Obiso, R.J., Jr., Azghani, A.O. and Wilkins, T.D. (1997a). The *Bacteroides fragilis* toxin fragilysin disrupts the paracellular barrier of epithelial cells. *Infec. Immun*. **65,** 1431–1439.

Obiso, R.J., Jr., Bevan, D. and Wilkins, T.D. (1997b). Molecular modeling and analysis of fragilysin, the *Bacteroides fragilis* toxin. *Clin. Infect. Dis*. **25,** S153–S155.

Obuch-Woszczatynski, P., Wintermans, R.G., van Belkum, A., Endtz, H., Pituch, H., Kreft, D., Meisel-Mikolajczyk, F. and Luczak, M. (2004). Enterotoxigenic *Bacteroides fragilis* (ETBF) strains isolated in the Netherlands and Poland are genetically diverse. *Acta Microbiol. Pol*. **53,** 35–39.

Onderdonk, A.B., Kasper, D.L., Cisneros, R.L. and Bartlett, J.G. (1977). The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: comparison of the pathogenic potential of encapsulated and unencapsulated strains. *Journal of Infectious Diseases* **136,** 82–89.

Pantosti, A., Cerquetti, M., Colangeli, R. and D'Ambrosio, F. (1994a). Detection of intestinal and extraintestinal strains of enterotoxigenic *Bacteroides fragilis* by the HT-29 cytotoxicity assay. *J. Med. Microbiol*. **41,** 191–196.

Pantosti, A., Piersimoni, C. and Perissi, G. (1994b). Detection of *Bacteroides fragilis* enterotoxin in the feces of a child with diarrhea. *Clin. Infect. Dis*. **19,** 809–810.

Pantosti, A., Menozzi, M.G., Frate, A., Sanfilippo, L., D'Ambrosio, F. and Malpeli, M. (1997). Detection of enterotoxigenic *Bacteroides fragilis* and its toxin in stool samples from adults and children in Italy. *Clin. Infect. Dis*. **24,** 12–16.

Polk, F.B. and Kasper, D.L. (1996). *Bacteroides fragilis* subspecies in clinical isolates. *Annals of Internal Medicine* **86,** 569–571.

Prindiville, T.P., Sheikh, R.A., Cohen, S.H., Tang, Y.J., Cantrell, M.C. and Silva, J., Jr. (2000). *Bacteroides fragilis* enterotoxin gene sequences in patients with inflammatory bowel disease. *Emerg. Infect. Dis*. **6,** 171–174.

Riegler, M., Lotz, M., Sears, C., Pothoulakis, C., Castagliuolo, I., Wang, C.C., Sedivy, R., Sogukoglu, T., Cosentini, E., Bischof, G., Feil, W., Teleky, B., Hamilton, G., LaMont, J.T. and Wenzl, E. (1999). *Bacteroides fragilis* toxin 2 damages human colonic mucosa *in vitro*. *Gut* **44,** 504–510.

Saidi, R.F. and Sears, C.L. (1996). *Bacteroides fragilis* toxin rapidly intoxicates human intestinal epithelial cells ($HT29/C_1$) *in vitro*. *Infec. Immun*. **64,** 5029–5034.

Saidi, R.F., Jaeger, K., Montrose, M.H., Wu, S. and Sears, C.L. (1997). *Bacteroides fragilis* toxin alters the actin cytoskeleton of HT29/C1 cells *in vivo* qualitatively but not quantitatively. *Cell Motility and Cytoskeleton* **37,** 159–165.

Salyers, A.A., Shoemaker, N.B., Stevens, A.M. and Li, L.Y. (1995). Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiological Reviews* **59,** 579–590.

Sanfilippo, L., Baldwin, T.J., Menozzi, M.G., Borriello, S.P. and Mahida, Y.R. (1998). Heterogeneity in responses by primary adult human colonic epithelial cells to purified enterotoxin of *Bacteroides fragilis*. *Gut* **43,** 651–655.

Sanfilippo, L., Li, C.K., Seth, R., Balwin, T.J., Menozzi, M.G. and Mahida, Y.R. (2000). *Bacteroides fragilis* enterotoxin induces the expression of IL-8 and transforming growth factor-beta (TGF-beta) by human colonic epithelial cells. *Clin. Exp. Immunol*. **119,** 456–463.

Saraste, M., Sibbard, P.R. and Wittinghofer, A. (1999). The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci*. **15,** 430–434.

Scotto d'Abusco, A.S., Sanfilippo, L., Menozzi, M.G. and Pantosti, A. (2000). Activity and role of BFT, an enterotoxin produced by *Bacteroides fragilis*. *J. Nat. Toxins*. **9,** 267–280.

Sears, C.L., Myers, L.L., Lazenby, A. and Van Tassell, R.L. (1995). Enterotoxigenic *Bacteroides fragilis*. *Clin. Infect. Dis*. **20***(Suppl 2)*, S142–S148.

Sears, C.L. (2001). The toxins of *Bacteroides fragilis*. *Toxicon* **39,** 1737–1746.

Smith, C.J., Tribble, G.D. and Bayley, D.P. (1998). Genetic elements of *Bacteroides* species: a moving story. *Plasmid* **40,** 12–29.

Tzianabos, A.O., Onderdonk, A.B., Rosner, B., Cisneros, R.L. and Kasper, D.L. (1993). Structural features of polysaccharides that induce intra-abdominal abscesses. *Science* **262,** 416–419.

Van Tassell, R.L., Lyerly, D.M. and Wilkins, T.D. (1992). Purification and characterization of an enterotoxin from *Bacteroides fragilis*. *Infection and Immunity* **60,** 1343–1350.

Van Tassell, R.L., Lyerly, D.M. and Wilkins, T.D. (1994a). Characterization of enterotoxigenic *Bacteroides fragilis* by a toxin-specific enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* **1,** 578–584.

Van Tassell, R.L., Lyerly, D.M. and Wilkins, T.D. (1994b). Production of antisera against the enterotoxin of *Bacteroides fragilis* and their use in a cytotoxicity neutralization assay of HT-29 cells. *Clinical and Diagnostic Laboratory Immunology* **1,** 473–476.

Vines, R.R., Perdue, S.S., Moncrief, J.S., Sentz, D.R., Barroso, L.A., Wright, R.L. and Wilkins, T.D. (2000). Fragilysin, the enterotoxin from *Bacteroides fragilis*, enhances the serum antibody response to antigen co-administered by the intranasal route. *Vaccine* **19,** 655–660.

Weikel, C.S., Grieco, F.D., Reuben, J., Myers, L.L. and Sack, R.B. (1992). Human colonic epithelial cells, HT29/$C_1$, treated with crude *Bacteroides fragilis* enterotoxin dramatically alter their morphology. *Infection and Immunity* **60,** 321–327.

Wells, C.L., Van De Westerlo, E.M.A., Jechorek, R.P., Feltis, B.A., Wilkins, T.D. and Erlandsen, S.L. (1996). *Bacteroides fragilis* enterotoxin modulates epithelial permeability and bacterial internalization by HT-29 enterocytes. *Gastroenterology* **110,** 1429–1437.

Werb, Z. (1997). ECM and cell surface proteolysis: regulating cellular ecology. *Cell* **91,** 439–442.

Werb, Z. and Yan Y (1998). A cellular striptease act. *Science* **282,** 1279–1280.

Wu, S., Lim, K.-C., Huang, J., Saidi, R.F. and Sears, C.L. (1998). *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc. Natl. Acad. Sci. USA* **95,** 14979–14984.

Wu, S., Dreyfus, L.A., Tzianabos, A.O., Hayashi, C. and Sears, C.L. (2002). Diversity of the metalloprotease toxin produced by enterotoxigenic *Bacteroides fragilis*. *Infection and Immunity* **70,** 2463–2471.

Wu, S., Morin, P.J., Maouyo, D. and Sears C.L (2003). *Bacteroides fragilis* enterotoxin induces c-Myc expression and cellular proliferation. *Gastroenterology* **124,** 392–400.

Wu, S., Powell, J., Mathioudakis, N., Kane, S., Fernandez, E. and Sears, C.L. (2004). *Bacteroides fragilis* enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor-kappaB pathway. *Infection and Immunity* **72,** 5832–5839.

Zhang, G., Svenungsson, B., Karnell, A. and Weintraub, A. (1999). Prevalence of enterotoxigenic *Bacteroides fragilis* in adult patients with diarrhea and healthy controls. *Clin. Infect. Dis.* **29,** 590–594.

CHAPTER

# 29

# Structure and mode of action of RTX toxins

*Albrecht Ludwig and Werner Goebel*

## INTRODUCTION

Many bacterial pathogens produce protein toxins that are able to insert into the plasma membrane of eukaryotic cells and generate transmembrane pores, thereby causing cell death and osmotic cell lysis. At low, sublytic concentrations, several of these pore-forming cytolysins subvert physiological functions of host cells by inducing specific cellular reactions. Pore-forming cytolysins thus represent important virulence factors that play central roles in the pathogenesis of bacterial infections.

Several different families of genetically, structurally, and functionally related bacterial pore-forming cytolysins have been identified. The RTX toxins, which are found in a variety of Gram-negative pathogens, represent one of the largest of these toxin families. The designation "RTX toxins" thereby refers to the presence of a characteristic and functionally important tandem array of glycine-rich nonapeptide repeats in these toxins (RTX stands for repeats in toxin).

## GENERAL CHARACTERISTICS OF RTX TOXINS

The RTX toxins are distinguished from other groups of pore-forming cytolysins by a number of common traits:

1. RTX toxins are large, single-chain protein toxins with molecular masses typically around 100 to 120 kDa (e.g., *Escherichia coli* α-hemolysin, 110 kDa). Some more distantly related members of this toxin family, such as adenylate cyclase toxin (AC toxin) from *Bordetella pertussis* and RtxA from *Vibrio cholerae* (VcRtxA), are even considerably larger (AC toxin, 177 kDa; (VcRtxA), 484 kDa).
2. RTX toxins contain several characteristic structural and functional elements, namely (i) a conserved hydrophobic domain in the N-terminal half of the toxin protein; (ii) a series of glycine-rich nonameric repeats in the C-terminal half of the toxin protein (the "repeat domain"); and (iii) a C-terminal uncleaved secretion signal. The consensus sequence of the nonameric repeats is GGXGXDXUX, where U is a large hydrophobic amino acid (often L, I, or F) and X is any amino acid. The number of repeats varies among different RTX toxins between less than 10 to about 40.
3. RTX toxins are synthesized as inactive protoxins that require posttranslational activation (modification) prior to export from the toxin-producing bacteria. This activation is directed by a specific accessory protein that is co-synthesized with the protoxin. So far, two RTX toxins, *E. coli* α-hemolysin and *B. pertussis* AC toxin, have been shown to be activated by covalent fatty acylation of specific lysine residues. It is assumed that the other members of this toxin family are activated by a similar mechanism.
4. Export of RTX toxins through the cell envelope of Gram-negative bacteria proceeds via the type I secretion pathway. The RTX toxins are thereby translocated in a single step across both the inner

and the outer membranes, without any detectable periplasmic intermediate. This is accomplished for each RTX toxin by a specific export apparatus, an ABC exporter, that is typically composed of three protein components (i) an inner membrane transport ATPase belonging to the superfamily of ATP-binding cassette (ABC) transporters, which provides energy for the secretory process; (ii) an adaptor protein also anchored in the inner membrane, which complexes with the ABC protein to form an inner membrane translocase; and (iii) a channel-forming outer membrane protein of the TolC family that is transiently recruited by the toxin-engaged inner membrane translocase for RTX toxin export. Interaction of the large periplasmic domain of the adaptor protein with the periplasmic domain of TolC or of a TolC homologue thereby results in the transient formation of a completely proteinaceous conduit that connects the cell cytosol to the external environment and that permits export of the corresponding RTX toxin. RtxA from *V. cholerae* and several other unusually large, closely related RTX toxins, which represent a subclass within the RTX toxin family, appear to be secreted by atypical four-component type I secretion systems that require two distinct ABC transporters.

5. The activity of the RTX toxins is $Ca^{2+}$-dependent. The calcium ions bind to the repeat domain, which thereby most likely adopts a very stable parallel β-roll structure that is required for productive interaction with target cells. $Ca^{2+}$ binding most likely occurs after toxin export.
6. RTX toxins usually generate short-lived, cation-selective pores (channels) in lipid membranes. The effective pore diameter appears to be uniform for a given RTX toxin, but it may vary among different RTX toxins. The mechanism of pore formation is unclear. RTX toxin pores or pore-forming structures have not as yet been detected by electron microscopy. Pore formation in the plasma membrane of target cells may lead to colloid osmotic cell lysis, apoptosis, or subversion of cell functions.
7. The genes specifically involved in synthesis and secretion of an RTX toxin are clustered and usually organized in a single operon. RTX toxin operons typically contain four contiguous genes designated *rtxC*, *rtxA*, *rtxB*, and *rtxD* in transcriptional order. *rtxA* is the structural gene of the toxin protein (i.e., of the protoxin), *rtxC* encodes the protein mediating the activation (fatty acylation) of the protoxin, and *rtxB* and *rtxD* encode the ABC protein and the adaptor protein, respectively, of the cognate ABC exporter. The gene encoding the outer membrane component of this secretion apparatus is in most cases unlinked and located elsewhere on the bacterial chromosome, consistent with the finding that TolC is involved in several different transport systems. Nevertheless, transport, some RTX toxin determinants (e.g., those encoding *B. pertussis* AC toxin and the RTX toxin of *Moraxella bovis*) include this gene downstream from *rtxD*. Furthermore, some RTX toxin gene clusters show deviations from the typical operon organization: (i) In the *B. pertussis* AC toxin gene cluster, the *rtxC* gene (which is referred to as *cyaC*) is inverted relative to the other genes and transcribed from its own promoter. (ii) The closely related RTX toxin gene clusters of *V. cholerae, Vibrio vulnificus* and several other species are composed of two divergently transcribed operons. One of these operons contains *rtxC* and *rtxA* downstream from a conserved hypothetical gene. The adjacent operon comprises *rtxB*, *rtxD*, and a third gene, *rtxE*, which encodes an additional ABC transporter that is related to RtxB.

## MEMBERS OF THE RTX TOXIN FAMILY

RTX toxins are produced by a broad range of Gram-negative bacteria, including *E. coli*, members of the family *Pasteurellaceae* (*Mannheimia*, *Pasteurella*, and *Actinobacillus* species), *B. pertussis*, *V. cholerae*, and other pathogens. Table 29.1 lists the most extensively studied and also several of the less closely characterized members of this toxin family, and shows their salient genetic and functional characteristics. Some of the latter RTX toxins were discovered only recently. It is therefore likely that additional members of this toxin family will be detected in the future.

Based on their target cell specificity, most of the RTX toxins can be subdivided into two categories, namely hemolysins and leukotoxins. RTX hemolysins such as *E. coli* α-hemolysin (HlyA) and *Actinobacillus pleuropneumoniae* ApxI are active against a wide range of cell types (erythrocytes, leukocytes, and others) from various species. RTX leukotoxins, such as LktA from *Mannheimia haemolytica* and LtxA from *Actinobacillus actinomycetemcomitans*, on the other hand, are toxic only to certain cell types (particularly leukocytes) in a species-specific fashion. Some of the RTX hemolysins (e.g., enterohemorrhagic *E. coli* [EHEC] hemolysin and ApxII from *A. pleuropneumoniae*) show clear limits in their target cell spectra, but still act against wider arrays of cells than the leukotoxins.

It is remarkable that in addition to the RTX toxins, several proteins with other activities and functions are

**TABLE 29.1** RTX toxins

| Bacterium | RTX toxin | Hemolytic activity | Cytotoxic activity | Size of toxin protein (kDa) | Operon structure[a] | Reference |
|---|---|---|---|---|---|---|
| *Escherichia coli* | α-Hemolysin (HlyA) | + | + | 110 | >*hlyCABD* | Felmlee *et al*. (1985) |
| | EHEC-Hemolysin (EHEC-HlyA) | + | + | 107 | >*hlyCABD* | Schmidt *et al*. (1995) |
| *Proteus vulgaris* | Hemolysin (HlyA) | + | n.d. | ~110 | >*hlyCABD*[b] | Welch (1987) |
| *Morganella morganii* | Hemolysin (HlyA) | + | n.d. | ~110 | >*hlyCABD*[b] | Koronakis *et al*. (1987) |
| *Mannheimia haemolytica* | Leukotoxin (LktA) | (+) | +[c] | 102 | >*lktCABD* | Lo *et al*. (1987) |
| *Mannheimia varigena* | Leukotoxin (PlLktA) | n.d. | + | 102 | >*pllktCABD*[d] | Chang *et al*. (1993) |
| *Pasteurella aerogenes* | PaxA | – | n.d. | 107.5 | >*paxCABD* | Kuhnert *et al*. (2000) |
| *Actinobacillus pleuro-pneumoniae* | ApxI | + | + | 110 | >*apxICABD* | Frey *et al*. (1991) |
| | ApxII | + | + | 102.5 | >*apxIICA*[e] | Chang *et al*. (1989) |
| | ApxIII | – | + | 113 | >*apxIIICABD* | Jansen *et al*. (1993a) |
| | ApxIV | + | n.d. | 202/170 | >ORF1*apxIVA*[f] | Schaller *et al*. (1999) |
| *Actinobacillus suis* | ApxI | + | + | 110 | >*apxICABD*[b] | Schaller *et al*. (2000) |
| | ApxII | + | + | 102.5 | >*apxIICA*[e] | Burrows and Lo (1992) |
| *Actinobacillus porci-tonsillarum* | ApxII | + | + | 102.5 | >*apxIICABD* | Kuhnert *et al*. (2005) |
| *Actinobacillus equuli* | AqxA | + | + | 110 | >*aqxCABD* | Berthoud *et al*. (2002) |
| *Actinobacillus actino-mycetemcomitans* | Leukotoxin (LtxA) | – | +[g] | 116 | >*ltxCABD* | Kraig *et al*. (1990) |
| *Bordetella pertussis* | AC toxin (CyaA) | + | + | 177 | *cyaC*< >*cyaABDE*[h] | Glaser *et al*. (1988) |
| *Vibrio cholerae* | RtxA (VcRtxA) | – | +[i] | 484 | *rtxArtxCchp*< >*rtxBDE*[j] | Lin *et al*. (1999) |
| *Vibrio vulnificus* | RtxA (VvRtxA) | n.d. | + | ~550 | *rtxArtxCchp*< >*rtxBDE*[j] | Boardman and Fullner Satchell (2004) |
| *Moraxella bovis* | Cytotoxin (MbxA) | + | + | 99 | >*mbxCABDtolC* | Angelos *et al*. (2003) |

[a]With exception of the *B. pertussis cya* and *M. bovis mbx* gene clusters, the gene encoding the outer membrane component of the toxin export apparatus is not linked to the corresponding *C*, *A*, *B*, and *D* genes, but located at a distant site. The transcriptional organization of the operons (5′ to 3′) is indicated by arrowheads.
[b]The gene cluster has not been sequenced.
[c]LktA from *M. haemolytica* is active only against ruminant leukocytes and platelets.
[d]*pllktB* and *pllktD* have not been sequenced.
[e]*apxIIB* and *apxIID* were apparently lost during evolution by deletion. Secretion of ApxII can, however, occur via ApxIB/ApxID.
[f]ORF1 appears to be required for activity of ApxIV, but it shares no significant homology with *rtxC* genes. Nothing is yet known regarding the secretion of ApxIV.
[g]LtxA from *Actinobacillus actinomycetemcomitans* is active only against human and primate leukocytes.
[h]*cyaC* is transcribed in opposite direction to *cyaABDE*; *cyaE* is homologous to *tolC* of *E. coli*.
[i]VcRtxA has no cytolytic activity, but causes depolymerization of F-actin by cross-linking G-actin.
jThe ORF provisionally named *chp* encodes a conserved hypothetical protein, and *rtxE* encodes an additional ABC transporter that is related to RtxB. *chp*, *rtxC*, and *rtxA* are transcribed in opposite direction relative to *rtxBDE*.
n.d., not determined.

also secreted from Gram-negative bacteria by type I secretion systems (i.e., by ABC exporters). These proteins do not share significant overall sequence similarity with the RTX toxins. However, most of them contain a series of glycine-rich $Ca^{2+}$-binding nonapeptide repeats that resemble those of the RTX toxins, and most of these proteins also possess an uncleaved C-terminal transport signal. Proteins exhibiting these features are classed with the RTX toxins in the superfamily of "RTX exoproteins." A large protein family belonging to the RTX exoproteins is the serralysin family of bacterial metalloproteases, which includes proteases from *Erwinia chrysanthemi* (Delepelaire and Wandersman, 1990), *Serratia marcescens* (Letoffe *et al*., 1991), *Pseudomonas aeruginosa* (Duong *et al*., 1996), and other bacteria. The RTX exoproteins further include related lipases from *S. marcescens* and *Pseudomonas fluorescens* (Duong *et al*., 1994; Akatsuka *et al*., 1995), surface layer (S-layer) proteins from *S. marcescens*, *Caulobacter crescentus*, and *Campylobacter rectus* (Awram and Smit, 1998; Kawai *et al*., 1998; Braun *et al*., 1999), the Fe-regulated proteins FrpA and FrpC of *Neisseria meningitidis* (Thompson and Sparling, 1993; Thompson *et al*., 1993), and the nodulation-signaling protein NodO from *Rhizobium leguminosarum* (Economou *et al*., 1990).

## PROPERTIES AND ACTIVITIES OF INDIVIDUAL RTX TOXINS

### *Escherichia coli* α-hemolysin (HlyA)

α-hemolysin (HlyA) from *E. coli* is the prototype member of the RTX toxin family. It is produced by a large percentage of the *E. coli* strains causing urinary tract and other extraintestinal infections, and contributes significantly to the virulence of these strains as shown in several animal models (Welch *et al*., 1981; Hacker *et al*., 1983; May *et al*., 2000).

*E. coli* α-hemolysin is synthesized as a protein of 110 kDa (1024 amino acids) that is posttranslationally activated with the aid of the co-synthesized protein HlyC (20 kDa) by covalent, quantitative fatty acylation of two lysine residues, Lys-564 and Lys-690 (Issartel *et al*., 1991; Stanley *et al*., 1994; Ludwig *et al*., 1996; Lim *et al*., 2000). Secretion of the activated toxin from the *E. coli* cell is accomplished by a specific ABC exporter that is composed of the inner membrane proteins HlyB (80 kDa) and HlyD (55 kDa) and the outer membrane protein TolC (51.5 kDa) (Wagner *et al*., 1983; Wandersman and Delepelaire, 1990; Thanabalu *et al*., 1998). The structural gene of α-hemolysin, *hlyA*, is clustered with the genes encoding HlyC, HlyB, and HlyD in the *hlyCABD* operon, while TolC is genetically unlinked (Felmlee *et al*., 1985; Hess *et al*., 1986). Structural and functional elements identified in the HlyA protein include (i) the hydrophobic domain (amino acids 238–410), which contains three particularly hydrophobic stretches (residues 238–259, 299–327, and 366–410) and which is involved in pore formation; (ii) the $Ca^{2+}$-binding repeat domain (amino acids 724–852) comprising 13 consecutive nonameric repeats; and (iii) the uncleaved transport signal located within the C-terminal 50–60 amino acids (Gray *et al*., 1986; Ludwig *et al*., 1988, 1991; Koronakis *et al*., 1989; Boehm *et al*., 1990b; Jarchau *et al*., 1994) (Figure 29. 1).

*E. coli* α-hemolysin shows a broad range of biological activities. At higher concentrations, it efficiently lyses erythrocytes from many species and displays strong cytotoxic and cytolytic activity against a variety of nucleated cells, including polymorphonuclear leukocytes (neutrophils) and monocytes (Cavalieri and Snyder, 1982; Bhakdi *et al*., 1989, 1990), T lymphocytes (Jonas *et al*., 1993), and tissue cells (Mobley *et al*., 1990; O'Hanley *et al*., 1991). α-hemolysin may thus directly promote survival of *E. coli* in the host by killing immune cells involved in first-line defense mechanisms, and it may further promote *E. coli* infections by causing local tissue damage. At low (sublytic) concentrations, *E. coli* α-hemolysin induces a broad range of reactions in host cells. In particular, it triggers the release of inflammatory lipid mediators (leukotrienes, hydroxyeicosatetraenoic acids) from leukocytes and platelets (König *et al*., 1986, 1990; Grimminger *et al*., 1991a), stimulates the secretion of pro-inflammatory cytokines such as interleukin (IL)-1 and IL-6 from monocytes and other cells (Bhakdi *et al*., 1990; May *et al*., 1996; Uhlen *et al*., 2000), and induces an oxidative burst and degranulation in neutrophils (Bhakdi *et al*., 1989; Bhakdi and Martin, 1991; Grimminger *et al*., 1991b). Furthermore, α-hemolysin stimulates the arachidonate metabolism and production of vasodilatory agents in endothelial cells and causes increased permeability of endothelial cell monolayers (Suttorp *et al*., 1990; Grimminger *et al*., 1997). It is assumed that the host cell reactions elicited by sublytic doses of α-hemolysin significantly contribute to the pathogenesis of extraintestinal *E. coli* infections.

EHEC hemolysin (EHEC-HlyA, EhxA), an RTX toxin that is specifically produced by EHEC strains, is approximately 60% identical in amino acid sequence to *E. coli* α-hemolysin (Schmidt *et al*., 1995; Bauer and Welch, 1996b). It has hemolytic and leukotoxic activity, but exhibits a stronger target cell specificity than α-hemolysin (Bauer and Welch 1996b). Sublytic concentrations of EHEC hemolysin have been shown to induce the production of IL-1β in human monocytes (Taneike *et al*., 2002). The role of EHEC hemolysin in the virulence of EHEC strains is, however, unclear.

### Leukotoxin of *Mannheimia haemolytica*

*M. haemolytica* (formerly *Pasteurella haemolytica*), the etiological agent of bovine and ovine pneumonic mannheimiosis (pasteurellosis), produces an RTX leukotoxin, LktA, that shows a rather narrow host and target cell specificity. Biological effects of this toxin are largely restricted to ruminant leukocytes and platelets (Kaehler *et al*., 1980; Shewen and Wilkie, 1982; Clinkenbeard and Upton, 1991). Nevertheless, LktA exhibits also weak hemolytic activity (Forrestier and Welch, 1990; Murphy *et al*., 1995). Synthesis and secretion of LktA are directed by the *lktCABD* operon (Lo *et al*., 1987; Strathdee and Lo, 1989). The amino acid sequence of LktA (102 kDa) is 36% identical to that of *E. coli* HlyA. Most strikingly, LktA has a shorter repeat domain than HlyA, comprising only eight nonapeptide repeats (Strathdee and Lo, 1987).

The effects of *M. haemolytica* leukotoxin on ruminant leukocytes are concentration-dependent. Exposure to high concentrations of LktA causes rapid swelling and oncosis (cytolysis) of bovine alveolar macrophages and neutrophils (Clinkenbeard *et al*., 1989a & b, Thumbikat *et al*., 2005), preventing effective phagocytosis and

bacterial killing by these cell types. At sublytic concentrations, LktA has pleiotropic effects: It provokes an oxidative burst and degranulation in bovine alveolar macrophages and neutrophils (Czuprynski *et al*., 1991; Maheswaran *et al*., 1993); it causes functional impairment of bovine lymphocytes (Czuprynski and Ortiz-Carranza, 1992; Hughes *et al*., 1994); it induces synthesis and release of chemotactic eicosanoids, such as leukotriene $B_4$ ($LTB_4$) and 5-hydroxyeicosatetraenoic acid in bovine neutrophils (Henricks *et al*., 1992; Clinkenbeard *et al*., 1994; Wang *et al*., 1999); and it induces the expression and secretion of inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], IL-1β) from bovine alveolar macrophages and peripheral blood neutrophils (Yoo *et al*., 1995; Leite *et al*., 2003). LktA may thus contribute to the severe inflammation that characterizes acute pneumonic mannheimiosis. Low concentrations of LktA can also induce bovine leukocytes to undergo apoptosis, which most likely plays a role in the initiation and persistence of *M. haemolytica* infections (Stevens and Czuprynski, 1996; Wang *et al*., 1998; Thumbikat *et al*., 2005). Several *in vivo* studies confirmed that LktA plays a central role in the pathogenesis of bovine pneumonic mannheimiosis (Petras *et al*., 1995; Tatum *et al*., 1998; Highlander *et al*., 2000).

LktA is also produced by strains of *Mannheimia glucosida* and *Pasteurella trehalosi*, but its role in infections caused by these species is less well documented (Davies *et al*., 2002). A leukotoxin related to LktA but exhibiting a broader species specificity has been identified in *Mannheimia varigena* (originally referred to as *Pasteurella haemolytica*-like bacterium) (Chang *et al*., 1993).

## RTX toxins of *Actinobacillus pleuropneumoniae* and closely related species

*A. pleuropneumoniae*, the causative agent of porcine pleuropneumonia, is extraordinarily versatile in the production of RTX toxins. Four different RTX toxins have been identified in this species. Three of these, ApxI, ApxII, and ApxIII, have been studied extensively. ApxI (110 kDa) is strongly hemolytic and strongly cytotoxic for phagocytic cells; its amino acid sequence is 56% identical to that of *E. coli* HlyA and 41% identical to that of *M. haemolytica* LktA (Frey *et al*., 1991, 1993b; Jansen *et al*., 1993b). ApxII (102.5 kDa) exhibits weak hemolytic and moderate cytotoxic activity, and is much more similar to *M. haemolytica* LktA (67% identity) than to ApxI (46% identity) and *E. coli* HlyA (47% identity); accordingly, ApxII contains only eight consecutive nonapeptide repeats (like LktA), while a series of 13 repeats is present in ApxI (Chang *et al*., 1989; Frey *et al*., 1991). ApxIII (113 kDa, 13 glycine-rich repeats in the repeat domain) shows strong cytotoxic activity against porcine alveolar macrohages and neutrophils, but it has no hemolytic activity. ApxIII is 50% identical to both ApxI and *E. coli* HlyA, and 41% identical to ApxII (Jansen *et al*., 1993a).

The different serotypes of *A. pleuropneumoniae* produce either ApxI plus ApxII (serotypes 1, 5, 9, and 11), ApxII plus ApxIII (serotypes 2, 3, 4, 6, and 8), only ApxI (serotype 10), or only ApxII (serotypes 7 and 12) (Kamp *et al*., 1991, 1994; Frey *et al*., 1992, 1993a; Jansen *et al*., 1993b, 1994). Interestingly, the virulence of these serotypes depends on which Apx toxins they synthesize (Frey, 1995). Serotypes secreting ApxI are exceptionally virulent and cause acute pleuropneumonia with high mortality, while those secreting only ApxII or ApxII plus ApxIII are usually moderately virulent and often cause chronic infections. Furthermore, serotypes that synthesize two different Apx toxins are generally more virulent than those producing just a single one, suggesting that these toxins synergize. Several studies confirmed that ApxI, ApxII, and ApxIII are major virulence factors of *A. pleuropneumoniae* and that ApxI particularly plays a critical role in the pathogenesis of acute pleuropneumonia (Cullen and Rycroft, 1994; Tascon *et al*., 1994; Jansen *et al*., 1995; Kamp *et al*., 1997).

While ApxI and ApxIII are encoded by conventional RTX toxin operons (*apxICABD* and *apxIIICABD*, respectively), the ApxII determinant is generally truncated in *A. pleuropneumoniae*, comprising only *apxIIC* and *apxIIA* (Jansen *et al*., 1992, 1993a, b, 1994; Frey *et al*., 1993a, b). *apxIIB* and *apxIID* were apparently lost during evolution. Nevertheless, all ApxII-producing *A. pleuropneumoniae* serotypes except serotype 3 can secrete this toxin via the ApxI exporter. In fact, with the exception of serotype 3, all serotypes that do not produce ApxI possess a truncated *apxI* operon comprising *apxIB* and *apxID*, and are consequently capable of producing the ApxI exporter which complements for secretion of ApxII (Jansen *et al*., 1992, 1993b; Frey *et al*., 1993a; Macdonald and Rycroft, 1993). The type I secretion system of ApxIII is apparently unable to export ApxII (Kuhnert *et al*., 2005).

ApxIV, the fourth RTX toxin of *A. pleuropneumoniae*, is encoded by a gene (*apxIVA*) that is apparently present in all *A. pleuropneumoniae* serotypes (Schaller *et al*., 1999). In contrast to ApxI–III, ApxIV is not produced in *A. pleuropneumoniae* cultures grown under various conditions *in vitro*, but serological data suggest that it is synthesized *in vivo*. ApxIV has weak hemolytic activity when expressed along with a putative gene referred to as ORF 1, which is located immediately upstream from *apxIVA*. ORF 1 shows, however, no significant homology to *rtxC* genes. Nothing is yet

known regarding the secretion of ApxIV. The ApxIV protein is unusually large (202 kDa in serotype 1, 170 kDa in serotype 3) and shows some variability in size due to differences in the $Ca^{2+}$-binding repeat domain (Schaller *et al*., 1999). The role of ApxIV in the pathogenesis of porcine pleuropneumonia remains to be elucidated.

Apx toxins have also been detected in *Actinobacillus* species that are closely related to *A. pleuropneumoniae*. *Actinobacillus suis*, an opportunistic pathogen that causes septicemia in young pigs, was shown to contain $apxICABD_{\text{var. suis}}$ and $apxIICA_{\text{var. suis}}$ gene clusters and to produce ApxI and ApxII (Burrows and Lo, 1992; Kamp *et al*., 1994; Schaller *et al*., 2000; Kuhnert *et al*., 2003). In *Actinobacillus rossii*, $apxIICA_{\text{var. rossii}}$ and $apxIIICABD_{\text{var. rossii}}$ were found, but only ApxII could be detected in cultures of this species (Schaller *et al*., 2000). *Actinobacillus lignieresii* was shown to contain an $apxICABD_{\text{var. lign.}}$ operon (Schaller *et al*., 2000), and a complete, functional *apxIICABD* operon was recently detected in *Actinobacillus porcitonsillarum* (Kuhnert *et al*., 2005). The equine pathogen *Actinobacillus equuli* produces, however, an RTX hemolysin referred to as Aqx (110 kDa) that is different from the Apx toxins (Berthoud *et al*., 2002; Kuhnert *et al*., 2003). PaxA (107.5 kDa) from *Pasteurella aerogenes*, on the other hand, shows high sequence similarity to ApxIII, and it appears also to be functionally similar to ApxIII since it is devoid of direct hemolytic activity (Kuhnert *et al*., 2000). Presence of the *paxCABD* operon and production of PaxA proved to be closely associated with *P. aerogenes* strains causing swine abortion and septicemia of newborn piglets, suggesting that PaxA is involved in the virulence of this species (Kuhnert *et al*., 2000).

## Leukotoxin of *Actinobacillus actinomycetemcomitans*

*A. actinomycetemcomitans* is the major pathogen in the etiology of aggressive periodontitis, especially of the localized form that affects young individuals, and has also been implicated in several other forms of periodontal disease, as well as in systemic infections. One of the putative virulence factors of this bacterium is the 116-kDa leukotoxin (LtxA), an RTX toxin exhibiting a unique specificity for human and some non-human primate leukocytes. In particular, LtxA selectively lyses primate monocytes/macrophages, neutrophils, and lymphocytes (Taichman *et al*., 1980; Tsai *et al*., 1984; Kelk *et al*., 2003). The monocytes and macrophages thereby proved to be most sensitive, and it has been suggested that these cells are lysed by a mechanism that involves activation of the cystein proteinase caspase 1 (Kelk *et al*., 2003). Primate neutrophils and lymphocytes are lysed only at 10-fold higher toxin concentrations. It has also been shown that LtxA can induce apoptosis in several cell types such as T lymphocytes (but apparently not in monocytes) (Mangan *et al*., 1991; Korostoff *et al*., 1998; Yamaguchi *et al*., 2001; Kelk *et al*., 2003). Due to these activities, LtxA may thus enable *A. actinomycetemcomitans* to evade detection by the host immune system. At low concentrations, LtxA triggers degranulation of neutrophils and induces production and secretion of IL-1β by human macrophages via activation of caspase 1 (Johansson *et al*., 2000; Kelk *et al*., 2005). It is remarkable that LtxA is more related to *E. coli* HlyA (51% identity) than to *M. haemolytica* LktA (43% identity) (Kraig *et al*., 1990).

The LtxA gene cluster, *ltxCABD* (also referred to as *lktCABD*), appears to be present in all *A. actinomycetemcomitans* strains, but the levels of LtxA expression vary considerably among different strains (Lally *et al*., 1989; Kraig *et al*., 1990; Hritz *et al*., 1996; Kolodrubetz *et al*., 1996). Localized aggressive and other forms of periodontitis are strongly associated with highly leukotoxic strains of *A. actinomycetemcomitans*, which is consistent with the hypothesis that LtxA plays an important role in the virulence of this bacterium (Zambon *et al*., 1983; Haraszthy *et al*., 2000).

Interestingly, LtxA expressed by rough (adherent) strains of *A. actinomycetemcomitans* remains associated with the bacterial cell, whereas smooth (nonadherent) strains secrete the toxin into the culture supernatants (Tsai *et al*., 1984; Lally *et al*., 1989; Kachlany *et al*., 2000). Ohta *et al*. (1993) suggested that retention of LtxA at the surface of *A. actinomycetemcomitans* may be due to electrostatic association of the toxin with nucleic acids bound to the bacterial cell surface. More recently, Kachlany *et al*. (2000) revealed that *tad* mutants of *A. actinomycetemcomitans* do not retain LtxA, but rather release it into the culture medium. The *tad* operon encodes long fibrils responsible for rough colony morphology and adherence to surfaces, which suggests that LtxA associates with a component of this adherence system, possibly the fibrils themselves (Kachlany *et al*., 2000). It has been shown that outer membrane vesicles secreted by *A. actinomycetemcomitans* are enriched in LtxA and that these vesicles can deliver the leukotoxin to target cells (Kato *et al*., 2002; Demuth *et al*., 2003).

## Adenylate cyclase toxin of *Bordetella pertussis*

*B. pertussis*, the causative agent of whooping cough, produces an RTX toxin known as adenylate cyclase toxin (AC toxin, CyaA) that is a major virulence factor required for the early stages of respiratory tract colonization

(Hewlett *et al*., 1976; Goodwin and Weiss, 1990; Gross *et al*., 1992; Khelef *et al*., 1994; Carbonetti *et al.,* 2005). AC toxin is expressed from the *cyaA* gene as a single 177-kDa protein (1706 amino acids), which is post-translationally activated by covalent fatty acylation through the co-synthesized protein CyaC (Glaser *et al*., 1988; Hewlett *et al*., 1989; Rogel *et al*., 1989; Barry *et al*., 1991; Hackett *et al*., 1994).

AC toxin is unique among the RTX toxins because it is a bifunctional toxin endowed with both cell-invasive adenylate cyclase activity and hemolytic (pore-forming) activity. The enzymatic and the hemolytic activity are thereby associated with different regions of the CyaA protein. In particular, the N-terminal about 400 amino acids of CyaA constitute the adenylate cyclase domain, while the amino acid sequence from position 400 to 1706 represents the hemolysin domain (Bellalou *et al*., 1990; Ehrmann *et al*., 1992; Sakamoto *et al*., 1992). Only the hemolysin moiety of CyaA exhibits sequence similarity to the other members of the RTX toxin family (Glaser *et al*., 1988), suggesting that the *cyaA* gene arose by fusion of an RTX toxin gene with an adenylate cyclase gene. The hemolysin domain of CyaA diverges significantly from the other RTX toxins (it is, for example, only 25% identical in sequence to *E. coli* HlyA), but it contains all structural elements typical of RTX toxins, namely (i) a hydrophobic domain suggested to be involved in channel formation (amino acids 500–700); (ii) a large $Ca^{2+}$-binding repeat domain (amino acids 1000–1600) containing almost 40 repeats with the consensus sequence GGXGXDXUX; and (iii) a C-terminal domain containing the secretion signal (amino acids 1600–1706) (Glaser *et al*., 1988) (Figure 29.1).

The principal pathophysiologic function of AC toxin is to cause rapid production of intracellular cyclic AMP (cAMP) following penetration of its catalytic domain into phagocytic cells, such as neutrophils and macrophages. This process is referred to as intoxication. In fact, relative to other RTX toxins, AC toxin is only weakly hemolytic (Bellalou *et al*., 1990; Rogel *et al*., 1991), which is consistent with the finding that it forms only very small pores in lipid bilayers (Benz *et al*., 1994b; Szabo *et al*., 1994). The hemolysin domain of CyaA is, nevertheless, required for the binding of AC toxin to target cells and for the subsequent translocation of the catalytic domain through the cell membrane into the host cell cytoplasm (Rogel *et al*., 1989; Bellalou *et al*., 1990; Bejerano *et al*., 1999). Once inside the cell, the AC domain is activated by binding of endogenous calmodulin, which enables it to catalyze the production of supraphysiologic levels of cAMP from host ATP (Wolff *et al*., 1980; Confer and Eaton, 1982; Rogel *et al*., 1991; Gray *et al*., 1998). Intoxication of immune effector cells leads to inhibition of chemotaxis, phagocytosis, and oxidative burst (Confer and Eaton, 1982; Friedman *et al*., 1987; Pearson *et al*., 1987; Weingart *et al*., 2000) and induces apoptosis of macrophages (Khelef and Guiso, 1995; Gueirard *et al*., 1998), thus allowing persistence of *B. pertussis* in the host and progression of the infection. Recently, it has also been reported that AC toxin induces the secretion of IL-6 by human tracheal epithelial cells (Bassinet *et al*., 2004).

The mechanism mediating the penetration of the catalytic domain into target cells has not been completely unraveled, but it is known that this process requires a negative membrane potential (Otero *et al*., 1995). In addition, several lines of evidence indicate that translocation of the AC domain and pore formation by the hemolysin domain of CyaA represent unrelated, if not mutually exclusive, events. Translocation of the catalytic domain through the target cell membrane depends, for example, on a conformational change of CyaA that is induced by $Ca^{2+}$ concentrations in the mM range (>200μM), while significantly lower $Ca^{2+}$ concentrations are required for membrane binding and hemolytic activity (Hewlett *et al*., 1991; Rogel and Hanski, 1992; Rose *et al*., 1995). In fact, the translocation-specific conformation of AC toxin inhibits its hemolytic activity (Gray *et al*., 2001). Furthermore, AC toxin induces intracellular cAMP production within

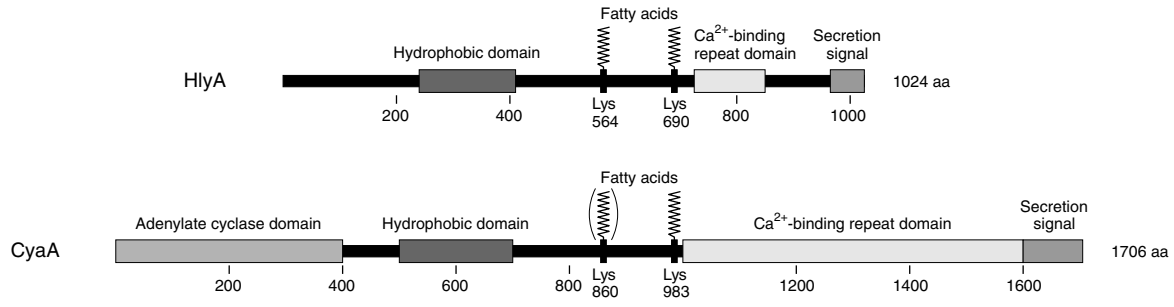



**FIGURE 29.1** Linear models of *E. coli* α-hemolysin (HlyA) and *B. pertussis* adenylate cyclase toxin (CyaA). Structural and functional domains are depicted as shaded boxes. aa: amino acids.

seconds to minutes after toxin addition, whereas hemolysis is observed only after much longer incubation times (Rogel *et al*., 1991; Otero *et al*., 1995; Gray *et al*., 1998). It has also been shown that AC toxin monomers are sufficient for intoxication, while hemolysis (pore formation) appears to be a highly cooperative event that likely involves oligomerization of membrane-embedded AC toxin molecules (Szabo *et al*., 1994; Gray *et al*., 1998; Osickova *et al*., 1999). Translocation of the AC domain into the host cell consequently cannot proceed through the transmembrane pore that is formed by the hemolysin moiety of CyaA. Khelef *et al*. (2001) suggested that internalization of AC toxin into endocytic vesicles contributes to macrophage cytotoxicity.

Unlike most other RTX toxins that are secreted into the extracellular milieu, AC toxin is not efficiently released by *B. pertussis* into the medium; the majority of the toxin rather remains associated with the bacterial surface after secretion (Hewlett *et al*., 1976). This is apparently due to physical interaction of AC toxin with the outer membrane filamentous hemagglutinin of *B. pertussis* (Zaretzky *et al*., 2002). Recently, Gray *et al*. (2004) revealed that the surface-bound AC toxin is unable to intoxicate target cells, and that only newly secreted AC toxin is responsible for intoxication. In addition, they found that delivery of the toxin requires close association of the bacteria with the target cells (Gray *et al*., 2004).

## RTX toxin of *Vibrio cholerae* and closely related RTX toxins of other bacteria

*V. cholerae* serotype O1 biotype El Tor produces an RTX toxin referred to as RtxA and VcRtxA that differs from all aforementioned RTX toxins with respect to both structure and mode of action. The protein deduced from the VcRtxA-encoding *rtxA* gene is 4,545 amino acids in length and has a predicted molecular mass of 484 kDa; VcRtxA is hence one of the largest single-polypeptide toxins known to date (Lin *et al*., 1999). Large regions of VcRtxA do not share sequence similarity with the typical RTX toxins. The presence of Gly- and Asp-rich (GD-rich), putatively calcium-binding repeats in the C-terminal region of VcRtxA, and the presence of genes located adjacent to *rtxA* that are related to the C, B, and D genes of other RTX toxin determinants indicated, nevertheless, that VcRtxA is indeed a member of the RTX toxin family. The GD-rich repeats of VcRtxA differ, however, from the nonameric repeats normally found in RTX toxins, because in the case of VcRtxA these repeats are elongated by nine additional residues. Hence, instead of the nonameric repeats, VcRtxA contains related GD-rich 18-residue repeats with the consensus sequence GGXGXDXX(V/I)XXGXXNXXX. Furthermore, unlike the typical RTX toxins, VcRtxA contains a series of glycine-rich 19-amino acid repeats with the consensus sequence GXAN(I/V)XT(K/H)VGDGXTVAVMX in its N-terminal region. These latter repeats do not show similarity to the GD-rich RTX repeats, and their function is unknown (Lin *et al*., 1999).

The gene cluster encoding VcRtxA differs from typical RTX toxin determinants in that it is composed of two divergently transcribed operons containing a total of six open reading frames (ORFs) (Lin *et al*., 1999; Boardman and Fullner Satchell, 2004). The first ORF in one of these operons encodes a 120-amino acid hypothetical protein. This ORF is followed by *rtxC* which encodes the protein that presumably mediates the maturation (fatty acylation) of the toxin, and the toxin structural gene *rtxA*. The adjacent operon encodes the type I secretion system for VcRtxA; it contains the genes *rtxB* and *rtxD*, which encode an ABC transporter (a transport ATPase) and an adaptor protein, respectively, and a third gene, *rtxE*, encoding another ABC transporter that is 60% similar in amino acid sequence to RtxB and that is required in addition to RtxB for export of VcRtxA (Boardman and Fullner Satchell, 2004). Interestingly, *V. cholerae* O1 strains of the classical biotype are unable to produce VcRtxA because they contain a 7.9-kb deletion within the *rtx* gene cluster that covers the 5′-terminal region of *rtxA*, all of *rtxC*, the hypothetical gene upstream from *rtxC*, and the 5′ end of *rtxB* (Lin *et al*., 1999).

In contrast to the conventional RTX toxins, VcRtxA is not associated with hemolytic or cytolytic activity (Lin *et al*., 1999). It rather causes depolymerization of F-actin stress fibers in a broad range of cell types, resulting in the rapid rounding of these cells in culture (Fullner and Mekalanos, 2000). VcRtxA accomplishes the F-actin depolymerization by cross-linking cellular actin monomers (G-actin) into dimers, trimers, and higher multimers, which suggests that disruption of the equilibrium between F-actin and G-actin, caused by the cross-linking of G-actin, might actually induce the depolymerization of the actin stress fibers (Fullner and Mekalanos, 2000). Recently, Sheahan *et al*. (2004) identified a 48-kDa domain within VcRtxA that covalently cross-links actin when transiently expressed within eukaryotic cells. Furthermore, they reported that deletion of this domain from VcRtxA completely abolishes the ability of the toxin to cross-link actin. These data suggested that VcRtxA inserts into the target cell plasma membrane and then, rather than forming a pore, delivers the actin cross-linking domain into the cell to either directly or indirectly catalyze the covalent cross-

linking of actin. Fullner *et al*. (2001, 2002) demonstrated that VcRtxA can disrupt the integrity of intestinal epithelial cell monolayers and that it stimulates pro-inflammatory immune responses, which may contribute to the pathogenesis of *V. cholerae* infections.

Recently, RTX toxins closely related to VcRtxA have been identified by genome sequence analysis in several other bacterial species, including the marine pathogen *Vibrio vulnificus* and the insect pathogens *Photorhabdus luminescens* and *Xenorhabdus bovienii* (Chen *et al*., 2003; Duchaud *et al*., 2003; Venter *et al*., 2004). The *rtxA*-like genes of all these bacteria encode toxins that are larger than 3500 amino acids and that share both the N-terminal 19-residue repeats and the atypical 18-residue RTX repeats. However, these toxins differ in central portions, suggesting that they may possess different cytotoxic activities (Sheahan *et al*., 2004; Boardman and Fullner Satchell, 2004). VcRtxA and RtxA from *V. vulnificus* (VvRtxA) are, for example, approximately 80 to 90% identical throughout most regions, but show extensive sequence divergence at two internal regions: (i) the VcRtxA sequence from residue 1963 to residue 2419, which contains the actin cross-linking domain, is absent in VvRtxA; instead, a different domain is present in VvRtxA at the same relative location; (ii) VvRtxA contains a domain at amino acid residue 3204 to 4095 that is missing in VcRtxA (Sheahan *et al*., 2004). Due to the presence of this latter domain, VvRtxA is even larger than VcRtxA (5,206 amino acids as deduced from the *V. vulnificus rtxA* gene). The absence of the actin cross-linking domain from VvRtxA indicates that this toxin does not cross-link actin. In fact, it has recently been shown that VvRtxA causes cell lysis (Sheahan *et al*., 2004).

An analysis of the DNA regions flanking the *rtxA*-like genes of the above-mentioned species revealed in all cases the presence of a gene cluster that resembles the *V. cholerae rtx* gene cluster (Chen *et al*., 2003; Duchaud *et al*., 2003; Boardman and Fullner Satchell, 2004; Venter *et al*., 2004). Thus, all of these bacteria carry two adjacent, divergently transcribed *rtx* operons with the toxin gene found in one of these two operons downstream from an *rtxC* homologue and a conserved hypothetical gene. The divergent operon generally comprises three genes encoding homologues of RtxB, RtxD, and RtxE. Interestingly, in the case of *P. luminescens*, five tandemly arranged *rtxA*-like genes were detected immediately downstream from the *rtxC*-like gene. Two of these toxin genes are intact while three are disrupted by frameshifts. Furthermore, three additional *rtxA* homologues (two complete genes and one disrupted by an insertion sequence) were found in another chromosomal region of *P. luminescens*. Thus, *P. luminescens* possesses eight *rtxA*-like genes, four of which are complete (Duchaud *et al*., 2003).

## SPECIFIC ASPECTS OF THE STRUCTURE AND MODE OF ACTION OF RTX TOXINS

### Posttranslational activation of RTX toxins

The activity of RTX toxins generally depends on a posttranslational activation (modification) that is mediated by the co-synthesized RtxC protein, as originally shown for *E. coli* α-hemolysin (HlyA) and *B. pertussis* AC toxin (CyaA) (Goebel and Hedgpeth, 1982; Barry *et al*., 1991; Hewlett *et al*., 1993). Issartel *et al*. (1991) revealed that *E. coli* HlyC activates α-hemolysin by catalyzing the acyl carrier protein (ACP)-dependent fatty acylation of the HlyA protein (pro-HlyA). Further studies demonstrated that pro-HlyA is covalently acylated at the ε-amino groups of two lysine residues, Lys-564 and Lys-690, and that the acylation at both sites is required for activity (Stanley *et al*., 1994; Ludwig *et al*., 1996). Under *in vitro* conditions, HlyC can acylate pro-HlyA with a range of fatty acids (Issartel *et al*., 1991). *In vivo*, however, HlyC exhibits a high selectivity for certain rather rare fatty acids. Mass spectrometric analysis of α-hemolysin produced by different *E. coli* strains particularly revealed that this toxin is heterogeneously acylated at both lysine residues with saturated 14-(68%), 15-(26%), and 17-(6%) carbon fatty acids (Lim *et al*., 2000).

Native AC toxin from *B. pertussis* strain 338 was shown to be covalently mono-acylated by a palmitoyl residue at Lys-983, which corresponds to Lys-690 of HlyA (Hackett *et al*., 1994). Furthermore, data from Basar *et al*. (2001) suggest that the acylation of Lys-983 is necessary and sufficient for the activation of this toxin. Nevertheless, recombinant AC toxin produced by *E. coli* in the presence of CyaC is acylated not only at Lys-983, but to some extent also at Lys-860, which is homologous to Lys-564 of *E. coli* HlyA (Hackett *et al*., 1995). In addition, recombinant AC toxin produced in *B. pertussis* strain 18323 was recently shown to be fully palmitoylated at both sites, Lys-983 and Lys-860, indicating that the extent of the acylation of Lys-860 depends on the strain and possibly its physiological state (Havlicek *et al*., 2001). Interestingly, *M. haemolytica* leukotoxin (LktA) contains only the lysine residue corresponding to Lys-564 of *E. coli* HlyA (Lys-554 in LktA), while a lysine residue homologous to Lys-690 of HlyA is missing. The acylation patterns of HlyA and LktA are consequently different, but the acylation process per se appears to be mechanistically similar for both toxins (Hormozi *et al*., 1998).

HlyC from *E. coli* has been shown to act as an acyltransferase that uses acylated ACP (acyl-ACP) as the fatty acid donor (Issartel *et al*., 1991; Trent *et al*., 1998;

Stanley *et al*., 1999). Furthermore, it has been demonstrated that HlyC binds the fatty acyl group from acyl-ACP to generate an acyl-HlyC intermediate, which subsequently transfers its fatty acyl group to pro-HlyA (Worsham *et al*., 2001, 2005). In the acylated HlyC intermediate, the fatty acid appears to be transiently bound to His-23 of HlyC (Worsham *et al*., 2001). Data from several laboratories indicate that the acylation of pro-HlyA by HlyC requires short amino acid sequences flanking the two acylation sites; these sequences most likely serve as independent HlyC recognition domains (Ludwig *et al*., 1996; Stanley *et al*., 1996; Langston *et al*., 2004). Other RtxC proteins show extensive sequence similarity to HlyC from *E. coli*, suggesting that they function in a similar way as HlyC.

## Secretion of RTX toxins

RTX toxins are secreted from Gram-negative bacteria via the type I secretion pathway. Toxin export thereby occurs in a single, energy-coupled step, resulting in direct passage of the toxin across both the inner and the outer cell membranes and the intervening periplasmic space. This is accomplished by the direct, reversible interaction of a toxin-specific inner membrane translocase, which is composed of two rotein components, with an outer membrane protein of the TolC family. When engaged by its export substrate (i.e., the corresponding RTX toxin), the translocase recruits TolC or a TolC homologue to form the active export complex containing all three proteins and the toxin. This assembly is transient: When the RTX toxin has been exported, the transport machinery disengages, and the components revert to their separate inner and outer membrane resting states (Thanabalu *et al*., 1998; Koronakis *et al*., 2000, 2004).

One of the components of the inner membrane translocase, the RtxB protein, belongs to the superfamily of ABC transporters (Higgins, 1992; Schmitt and Tampe, 2002), since it contains a nucleotide-binding domain that provides energy for the export process by hydrolysis of ATP. The other component of the translocase, the RtxD protein, is a member of the putative membrane fusion protein (MFP) family (Dinh *et al*., 1994) and functions as an adaptor protein that enables the translocase to interact in a reversible manner with TolC or a TolC homologue. Together, these three proteins form a secretion apparatus that is referred to as ABC exporter. Type I secretion of RTX toxins depends on the presence of an uncleaved secretion signal at the C-terminus of the toxin protein (Gray *et al*., 1986; Koronakis *et al*., 1989; Iwaki *et al*., 1995). The activation (fatty acylation) of RTX toxins is not required for secretion (Ludwig *et al*., 1987).

The *E. coli* α-hemolysin secretion apparatus has been studied in detail. HlyB, the inner membrane ABC protein of this ABC exporter, has been shown to contain an N-terminal integral membrane domain with six or eight predicted transmembrane segments and a C-terminal cytoplasmic nucleotide-binding domain (Wang *et al*., 1991; Gentschev and Goebel, 1992; Schmitt *et al*., 2003). HlyD, the adaptor protein, is anchored in the cytoplasmic membrane by a single transmembrane helix that links a small N-terminal cytoplasmic domain to a large C-terminal domain located in the periplasm (Wang *et al*., 1991; Schülein *et al*., 1992). This periplasmic domain which is predicted to contain long α–helices, most likely interacts with TolC to form the complete transport complex (Schlör *et al*., 1997; Thanabalu *et al*., 1998; Johnson and Church, 1999).

The analysis of the three-dimensional (3D) structure of TolC demonstrated that TolC is a trimeric outer membrane protein. In particular, the three TolC protomers assemble to form a 140-Å-long hollow continuous conduit, a "channel-tunnel," that spans both the outer membrane and the periplasmic space (Koronakis *et al*., 2000). This conduit consists of an α-helical barrel, which forms a 100-Å-long tunnel through the periplasm, and of a contiguous 12-stranded β-barrel, which forms a 40-Å-long channel through the outer membrane. The periplasmic α-helical barrel is a structure in which twelve α-helices (four from each TolC monomer) are packed in an antiparallel arrangement to form a hollow cylinder. The interior diameter of the central pore of the TolC trimer is 19.8 Å throughout the outer membrane channel and most of the tunnel, with the channel exit being constitutively open. In the resting state, the periplasmic entrance of the α-helical barrel is, however, constricted by three α-helices that are twisted inwards. Accordingly, TolC assembled in planar lipid bilayers causes only small conductance increases (Andersen *et al*., 2002a). When TolC is recruited for α-hemolysin export, the entrance of the α-helical barrel is opened for the toxin to gain access to the exit duct (Eswaran *et al*., 2003). This is apparently achieved by an iris-like realignment (untwisting) of the tunnel entrance helices (Andersen *et al*., 2002b). Interaction of the HlyA-engaged HlyB/HlyD complex with the TolC channel-tunnel thus results in the assembly of a continuous, completely proteinaceous conduit, that transiently connects the cell cytosol to the external environment and that specifically permits the export of α-hemolysin. HlyD has been shown to trimerize in the inner membrane, which is in line with the finding that this protein directly interacts with the TolC trimer (Thanabalu *et al*., 1998; Balakrishnan *et al*., 2001). HlyB, on the other hand, has been shown to act as a dimer (Benabdelhak *et al*., 2005; Zaitseva *et al*., 2005). The

number of HlyB dimers present in the HlyB/HlyD complex remains, however, to be determined.

The C-terminal secretion signal of *E. coli* α-hemolysin is apparently recognized by HlyB (Oropeza-Wekerle *et al*., 1990; Sheps *et al*., 1995). Moreover, it has recently been suggested that the C-terminus of HlyA is specifically recognized by the nucleotide-binding domain of HlyB and that the secretion is initiated by the subsequent displacement of HlyA from HlyB by ATP (Benabdelhak *et al*., 2003). Consistent with this, it has been shown that binding and hydrolysis of ATP by HlyB is directly coupled to the export of HlyA (Koronakis *et al*., 1995). Interaction of HlyA with HlyB alone is, however, insufficient to induce the export of HlyA. In fact, several studies suggest that HlyA must also bind to the cytosolic domain of HlyD to trigger recruitment of TolC and assembly of the type I export complex (Thanabalu *et al*., 1998; Pimenta *et al*., 1999; Balakrishnan *et al*., 2001). In addition, the proton motive force was found to be required at an early stage of HlyA secretion (Koronakis *et al*., 1991).

As noted above, it has recently been shown that the type I secretion system of the *V. cholerae* RTX toxin (VcRtxA) requires two distinct transport ATPases, namely RtxB and RtxE. Based on the finding that HlyB of *E. coli* acts as a homodimer, Boardman and Fullner Satchell (2004) suggested that RtxB and RtxE may function as a heterodimer. Furthermore, they speculated that the large size of VcRtxA may require multiple contact points within the structurally diverse regions of RtxB and RtxE to enhance specificity, thereby necessitating two distinct transport ATPases. Consistent with this hypothesis, other unusually large RTX toxins that are closely related to VcRtxA, such as the RTX toxins from *V. vulnificus, P. luminescens*, and *X. bovienii*, also appear to be exported by four-component type I secretion systems requiring two distinct ABC transporters in addition to an adaptor protein and an outer membrane protein of the TolC family.

The structural characteristics of the C-terminal secretion signals of RTX toxins are not entirely understood. In fact, RTX toxins generally show little sequence similarity in their C-terminal regions. Several studies indicated that the transport signals of RTX toxins are at least partially defined by higher-order structures (Hess *et al*., 1990; Stanley *et al*., 1991; Zhang *et al*., 1993, 1995), while others suggested that a small number of individual amino acid residues within the C-terminal region are critical for the interaction with the transport apparatus (Kenny *et al*., 1994; Chervaux and Holland, 1996). Recently, Hui and colleagues analyzed the functional elements of the *E. coli* α-hemolysin signal sequence using a combinatorial approach and revealed that the secondary structure and general biophysical properties rather than the primary sequence are important (Hui *et al*., 2000; Hui and Ling, 2002). In particular, they identified two functional domains within the secretion signal of HlyA. In the first domain (residues -49 to -28 with respect to the C-terminus), an amphiphilic α-helix extending from residue -49 to -38 was found to be critical, while in the second domain (last eight residues of HlyA), the relative lack of positively charged residues (i.e., hydrophobicity) proved to be important.

## Binding of calcium ions by RTX toxins

RTX toxins require $Ca^{2+}$ ions for activity, as shown first for *E. coli* α-hemolysin (Short and Kurtz, 1971) and *B. pertussis* AC toxin (Hewlett *et al*., 1991; Rogel and Hanski, 1992). Several studies revealed that the calcium ions bind to the nonapeptide repeats with the consensus sequence GGXGXDXUX in the C-terminal half of these toxins (Ludwig *et al*., 1988; Boehm *et al*., 1990b; Rose *et al*., 1995; Rhodes *et al*., 2001).

Analysis of the 3D structure of alkaline protease (AprA) from *P. aeruginosa* (one of the non-toxin RTX exoproteins) demonstrated that the consecutive nonameric repeats constitute a novel $Ca^{2+}$-binding structure called parallel β-roll, in which consecutive β-strands are wound in a right-handed helix (Baumann *et al*., 1993). The calcium ions are thereby bound within the turns connecting the β-strands. In particular, the first six residues of each repeat motif (GGXGXD) form a turn that binds a $Ca^{2+}$ ion, while the remaining three residues (XUX) build a short β-strand. One complete turn of the parallel β-roll thus consists of two consecutive nine-residue repeats (Baumann *et al*., 1993; Lilie *et al*., 2000). 3D structures of RTX toxins have not yet been determined, but it is assumed that their repeat domains likewise fold into parallel β-rolls upon binding of calcium ions.

The repeats in the extraordinarily large repeat domain of *B. pertussis* CyaA are organized in five cassettes (Glaser *et al*., 1988; Rhodes *et al*., 2001). Indeed, this repeat domain apparently folds into five parallel β-rolls (β-sheet helices), all of which appear to be required for cell intoxication (Rhodes *et al*., 2001). Interestingly, it has been shown that the repeat domain of CyaA contains a small number (three to five) of high-affinity $Ca^{2+}$-binding sites and about 35 low-affinity $Ca^{2+}$-binding sites (Rose *et al*., 1995; Rhodes *et al*., 2001). Rose *et al*. (1995) suggested that binding of calcium ions to the high-affinity sites might be required for the binding of CyaA to cell membranes and for lysis of erythrocytes, while binding of $Ca^{2+}$ to the low-affinity sites might induce the large conformational change that is required for entry of the catalytic domain into target cells.

Binding of the calcium ions to the repeats and formation of the parallel β-roll(s) most likely occurs after secretion of the RTX toxins, i.e., outside the toxin-producing bacteria, because the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in these bacteria is generally very low. In *E. coli*, for example, $[Ca^{2+}]_i$ is tightly regulated to a constant level of only about 0.1 μM (Gangola and Rosen, 1987). *E. coli* HlyA requires, however, $Ca^{2+}$ concentrations of >100 μM for full hemolytic activity (Ostolaza *et al*., 1995; Döbereiner *et al*., 1996). Thus, as long as HlyA is within the *E. coli* cell, its repeat domain is presumably flexible, which is probably a prerequisite for secretion through the *E. coli* cell envelope. After export, the calcium ions present in the extracellular medium may then bind to the repeat domain and thereby trigger the rapid formation of the stable β-roll structure, thus inducing the toxin to adopt a well-defined tertiary structure. The binding of calcium ions in the external medium may generally facilitate the folding of RTX exoproteins following secretion in an at least partially unfolded or extended conformation (Baumann *et al*., 1993).

## Binding of RTX toxins to target cells

The interaction of RTX toxins with eukaryotic cell membranes can be subdivided into two stages, namely (i) binding of the toxin to the membrane surface (adsorption), and (ii) membrane insertion resulting in pore formation. Significant progress has been made in the past years towards identifying cell surface receptors of RTX toxins. In particular, it has been repeatedly shown that $\beta_2$ integrins play an important role in binding of RTX toxins to leukocytes. $\beta_2$ integrins are leukocyte-specific, heterodimeric cell surface glycoproteins sharing the same $\beta_2$ subunit, CD18, but containing different α subunits. CD18 particularly associates with the α chains $\alpha_L$ (CD11a), $\alpha_M$ (CD11b), $\alpha_X$ (CD11c), and $\alpha_D$ (CD11d), to give rise to four distinct $\beta_2$ integrins: $\alpha_L\beta_2$ (CD11a/CD18), also known as lymphocyte function-associated antigen 1 (LFA-1); $\alpha_M\beta_2$ (CD11b/ CD18), also referred to as macrophage antigen 1 (Mac-1) and complement receptor 3 (CR3); $\alpha_X\beta_2$ (CD11c/CD18), also known as CR4 and p150/95; and $\alpha_D\beta_2$ (CD11d/ CD18) (Arnaout, 1990).

Lally and coworkers identified LFA-1 (CD11a/ CD18) as a receptor for *A. actinomycetemcomitans* leukotoxin (LtxA), as well as for *E. coli* α-hemolysin on the surface of human leukocytes (Lally *et al*., 1997). In addition, they suggested recently that LtxA recognizes a sequence or structure in the N-terminal β-propeller domain of human CD11a that is not present in the murine counterpart, which might explain the selective activity of LtxA against human (primate) leukocytes. AC toxin from *B. pertussis* has been shown to use the $\alpha_M\beta_2$ integrin (CD11b/CD18) as a specific host cell receptor, obviously by interacting with CD11b (Guermonprez *et al*., 2001). CD11b is exclusively expressed in immune cells such as neutrophils, macrophages, dentritic cells, and natural killer cells, which is consistent with the finding that phagocytes are the primary target cells of AC toxin *in vivo*. El-Azami-El-Idrissi *et al*. (2003) suggested that the main CD11b-interacting domain of CyaA is located within the glycine-rich repeat region, specifically between the amino acids 1166 and 1281. LktA from *M. haemolytica* apparently binds to bovine CD18, which enables this leukotoxin to use all $\beta_2$ integrins as receptors on bovine leukocytes (Wang *et al*., 1998; Li *et al*., 1999; Jeyaseelan *et al*., 2000; Deshpande *et al*., 2002; Dileepan *et al*., 2005). Leite *et al*. (2000, 2002, 2003) revealed that inflammatory cytokines secreted from bovine neutrophils in response to activation by LktA induce an up-regulation of LFA-1 in these cells, which in turn enhances LktA binding and consequently augments LktA cytotoxicity. In addition, they found that IL-1β (and perhaps other inflammatory cytokines) released from bovine peripheral blood mononuclear cells following infection with bovine herpesvirus-1 (BHV-1) can stimulate LFA-1 expression in these mononuclear cells and in bystander bovine neutrophils, thus enhancing LktA binding and LktA-mediated cytotoxicity (Leite *et al*., 2004, 2005). Consistent with these results, Yamaguchi *et al*. (2004) reported that TNF-α and IL-1 enhance *A. actinomycetemcomitans* LtxA-induced cell apoptosis by stimulating the expression of LFA-1.

Whether specific receptors are involved in or required for the binding of RTX toxins to erythrocytes is unclear. Bauer and Welch (1996a) found that the number of binding sites for *E. coli* HlyA on sheep erythrocytes is limited to a few thousand, which suggested that HlyA interacts with a specific cell surface component. Cortajarena *et al*. (2001, 2003) reported that glycophorin acts as a receptor for *E. coli* HlyA on red blood cells and that a region near the C-terminus of HlyA (amino acids 914–936) is essential for the toxin to bind to glycophorin. Nevertheless, other investigators did not observe saturability of the binding of HlyA to erythrocytes, suggesting that the binding is not receptor-dependent (Eberspächer *et al*., 1989). Furthermore, it has recently been suggested that erythrocytes do not contain specific receptors for *B. pertussis* AC toxin (Martin *et al*., 2004).

## Pore formation by RTX toxins

Osmotic protection experiments performed with *E. coli* α-hemolysin yielded first clues that RTX toxins may

damage target cell membranes by generating transmembrane pores (Bhakdi *et al*., 1986). Subsequently, biophysical studies such as planar lipid bilayer experiments and patch-clamp experiments demonstrated that RTX toxins form cation-selective membrane pores (channels) that have a defined size and a short lifetime of only a few seconds (Menestrina *et al*., 1987, 1996; Benz *et al*., 1989, 1994a, b; Szabo *et al*., 1994; Lear *et al*., 1995; Maier *et al*., 1996; Schmidt *et al*., 1996; Karakelian *et al*., 1998). Based on the single-channel conductance, an effective pore diameter in the range of 1 to 3 nm was calculated for *E. coli* α-hemolysin and several other RTX toxins, but some RTX toxins, such as *B. pertussis* AC toxin and *A. pleuropneumoniae* ApxIII, were found to generate considerably smaller pores. It is remarkable that, despite the large molecular mass of the RTX toxins, target membranes attacked by high doses of these toxins do not exhibit recognizable pore structures when examined by electron microscopy (Bhakdi *et al*., 1986; Soloaga *et al*., 1999). The reason for this is unclear, but the instability of the RTX toxin pores might play a role.

Structure/function analyses of several RTX toxins (*E. coli* HlyA, *B. pertussis* CyaA, *M. haemolytica* LktA) revealed that the highly conserved hydrophobic domain in the N-terminal half of these toxins is critical for their capability to generate transmembrane pores. In particular, it has been shown that deletions in this region and mutations altering their hydrophobicity specifically abolish or impair the cytolytic and pore-forming activity (Ludwig *et al*., 1987, 1991; Glaser *et al*., 1988; Cruz *et al*., 1990). In addition, it has been demonstrated that certain peptides representing parts of the hydrophobic region of *E. coli* HlyA are able to form pores in artificial lipid bilayers. One of these peptides also exhibited hemolytic activity (Oropeza-Wekerle *et al*., 1992). Two recent studies employing cystein-scanning mutagenesis and membrane insertion-dependent labeling, respectively, further confirmed that the hydrophobic domain of *E. coli* HlyA is the principal region of this toxin that inserts into the target membrane (Hyland *et al*., 2001; Schindel *et al*., 2001). Computer-assisted structure predictions suggested that the hydrophobic region of HlyA contains both hydrophobic and amphipathic α-helices that are long enough to span a lipid bilayer (Ludwig *et al*., 1991). Thus, this region appears to be directly involved in the generation of the pore. Insertion of *E. coli* HlyA and *B. pertussis* CyaA into target membranes has also been shown to be associated with a change in toxin conformation (Moayeri and Welch, 1997; Osickova *et al*., 1999).

Pore-forming exotoxins are usually synthesized as water-soluble (hydrophilic) molecules that have the intrinsic capability to spontaneously insert into target membranes and hence to adopt an amphiphilic structure. With respect to the mechanism of membrane insertion, two basic strategies have been recognized among membrane-inserting toxins:

(1) Toxins that do not contain extended hydrophobic sequences, such as *Staphylococcus aureus* α-toxin, the cholesterol-dependent cytolysins, and anthrax toxin protective antigen, must oligomerize after binding to the target membrane; subsequently, amphipathic sequences of the oligomerized toxin molecules associate with each other and insert as β-hairpins into the membrane, resulting in the formation of a stable β-barrel, which represents a transmembrane channel lined by antiparallel β-strands. In the β-barrel, the β-hairpins are arranged in such a way that their hydrophilic residues are oriented towards the lumen of the channel, while the intervening hydrophobic residues face the membrane lipids (Song *et al*., 1996; Petosa *et al*., 1997; Shatursky *et al*., 1999).
(2) Toxins such as the pore-forming colicins and diphtheria toxin contain hydrophobic and amphipathic α-helices long enough to span a lipid membrane. The hydrophobic α-helices are thereby buried within the toxin as long as it is in its water-soluble form. However, at the target membrane, partial unfolding of the toxin leads to a change in toxin conformation and insertion of the hydrophobic (and likely of amphipathic) α-helices as α-helical hairpins into the membrane, perhaps resulting in the generation of a transmembrane pore; membrane insertion thereby may or may not require toxin oligomerization (Lesieur *et al*., 1997; Stroud *et al*., 1998; D'Silva and Lala, 2000).

In the case of the RTX toxins, the mechanism of membrane insertion and pore formation is unknown. Nevertheless, based on the data described above, it is tempting to speculate that these toxins insert into membranes by a mechanism that might be similar to that employed by the latter group of toxins.

Whether pore formation by the RTX toxins is dependent on a toxin oligomerization is unclear. In fact, RTX toxins have been recovered only in monomeric form from target membranes, and it has been repeatedly suggested that these toxins act as monomers (Menestrina *et al*., 1987; Eberspächer *et al*., 1989; Stanley *et al*., 1993). On the other hand, dose-response analyses indicated that the lysis of target cells by RTX toxins is a highly cooperative event that likely involves toxin oligomerization (Cavalieri and Snyder, 1982; Simpson *et al*., 1988; Bhakdi *et al*., 1989; Taichman *et al*., 1991; Bauer and Welch, 1996b; Gray *et al*., 1998). In

the case of *E. coli* HlyA and *B. pertussis* CyaA, successful complementation between different inactive toxin mutants has been observed, which further argues in favor of the formation of toxin oligomers (Ludwig *et al*., 1993; Iwaki *et al*., 1995; Bejerano *et al*., 1999). In addition, the biophysical characteristics of RTX toxin pores are compatible with the hypothesis that these pores are generated by transiently formed (unstable) toxin oligomers (Benz *et al*., 1989).

Some investigators suggested that the cytolytic activity of RTX toxins might be due to a mechanism different from the generation of discrete-sized transmembrane pores. Moayeri and Welch (1994) proposed that *E. coli* HlyA creates membrane lesions that increase in size over time. Soloaga *et al*. (1999) suggested that HlyA inserts only into the outer leaflet of the lipid bilayer through a number of amphipathic α-helices, thereby causing destabilization of the membrane and transient bilayer breakdown.

## Role of fatty acylation and $Ca^{2+}$ binding for the activity of RTX toxins

RTX toxins require both the fatty acylation and the binding of calcium ions to the glycine-rich repeats for all known cytotoxic activities. The actual functions of these modifications are, however, not completely understood. Pore formation by *E. coli* HlyA in artificial lipid bilayers is apparently independent of $Ca^{2+}$ and not affected by deletions in the repeat domain (Ludwig *et al*., 1988; Döbereiner *et al*., 1996). In addition, nonacylated HlyA (pro-HlyA) forms pores in planar lipid bilayers, which have similar properties as those generated by the acylated toxin, although the pore-forming activity of pro-HlyA is considerably reduced compared to that of HlyA (Ludwig *et al*., 1996). Thus, $Ca^{2+}$ binding and acylation appear to be critical for the interaction between HlyA and eukaryotic cell membranes at some stage preceding pore formation. Several studies indicated that $Ca^{2+}$ binding and acylation are required for the binding of RTX toxins to target cell membranes (Ludwig *et al*., 1988, 1996; Boehm *et al*., 1990a, b; Issartel *et al*., 1991; Gray *et al*., 1999), even though this was not confirmed in some other studies (Bauer and Welch, 1996a; Soloaga *et al*., 1996; Bakas *et al*., 1998). Recent evidence indicates that nonacylated RTX toxins may loosely bind to target cells, but that the fatty acylation is essential for the tight and productive interaction that is required for cytotoxic effects to occur (Sun *et al*., 1999; El-Azami-El-Idrissi *et al*., 2003; Thumbikat *et al*., 2003). Lee *et al*. (2005) revealed that AC toxin from *B. pertussis* undergoes self-association in solution, and they suggested that this process requires the posttranslational acylation of the toxin, but not calcium.

## Role of LPS in RTX toxin structure and function

Preparations of active, extracellular RTX toxins typically contain substantial amounts of lipopolysaccharides (LPS). Indeed, some RTX toxins, such as *E. coli* α-hemolysin and *M. haemolytica* leukotoxin, have been shown to complex with LPS (Bohach and Snyder, 1985; Czuprynski and Welch, 1995; Li and Clinkenbeard, 1999). LPS is not required for the cytotoxic and cytolytic activity of RTX toxins (Welch, 2001). Nevertheless, the physical interaction between LPS and RTX toxins is functionally important, because LPS indirectly enhances RTX toxin activities. It has been particularly suggested that LPS is important for stabilizing the active conformation of *E. coli* HlyA and/or for protecting it from aggregation or degradation (Stanley *et al*., 1993; Bauer and Welch, 1997; Herlax *et al*., 2005). Consistent with this, it has been reported that complexation between LPS and *M. haemolytica* LktA results in enhanced and stabilized leukolytic activity of LktA, and that LPS also enhances inflammatory cytokine induction in bovine alveolar macrophages exposed to low doses of this leukotoxin (Li and Clinkenbeard, 1999; Lafleur *et al*., 2001). Recently, Ross *et al*. (2004) revealed that *B. pertussis* AC toxin synergizes with LPS to promote IL-10 production.

## Importance of calcium as a signaling molecule in the action of RTX toxins

Jorgensen *et al*. (1983) revealed that *E. coli* α-hemolysin causes changes in the selective permeability of the cytoplasmic membrane of erythrocytes, resulting in the rapid influx of extracellular $Ca^{2+}$ ions and in the efflux of $K^+$ ions. Subsequent studies showed that several other RTX toxins, such as *M. haemolytica* LktA and *A. actinomycetemcomitans* LtxA, also cause an unregulated $Ca^{2+}$ influx into target cells (Taichman *et al*., 1991; Sun *et al*., 1999). This calcium influx most likely plays an important role in the killing of host cells by RTX toxins. A popular hypothesis is that it initiates cytoskeletal destruction with multiple necrotic sequels that promote colloidal osmotic cell lysis (Welch, 2001).

Calcium is central as an intracellular second messenger and is involved in the regulation of a diverse array of cell functions (Berridge *et al*., 1998). Recent findings indicate that the $Ca^{2+}$ influx evoked by sublytic doses of RTX toxins is involved in the RTX toxin-mediated induction of inflammatory responses, particularly by causing activation of nuclear factor (NF)-κB. NF-κB is one of the major transcription factors required for induction of inflammatory responses in various cell types (Baeuerle and Baltimore, 1996). Several

pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, are known to be regulated by this protein. In non-stimulated cells, NF-κB is bound to a cytoplasmic inhibitor protein, I-κB, which prevents its translocation into the nucleus. NF-κB activation results in degradation of I-κB, leaving NF-κB free to translocate into the nucleus and to induce the expression of genes involved in inflammatory responses (Guha and Mackman, 2001). Hsuan *et al*. (1999) observed that sublytic concentrations of *M. haemolytica* LktA induce nuclear translocation of NF-κB and expression of TNF-α, IL-1β, and IL-8 in bovine alveolar macrophages, and that elevation of the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) is a prerequisite for NF-κB activation and expression of cytokine genes. The LktA-induced production of leukotriene $B_4$ by bovine neutrophils was also found to be dependent on a sustained and excessive increase in $[Ca^{2+}]_i$ (Cudd *et al*., 2003a). Elevation of $[Ca^{2+}]_i$ in LktA-stimulated bovine alveolar macrophages and neutrophils seems to be due to both influx of extracellular $Ca^{2+}$ through voltage-gated calcium channels (Ortiz-Carranza and Czuprynski, 1992; Hsuan *et al*., 1998) and release of calcium from intracellular stores within the endoplasmatic reticulum (ER) (Cudd *et al*., 2003b).

Uhlen *et al*. (2000) revealed that sublytic concentrations of *E. coli* α-hemolysin induce low-frequency $[Ca^{2+}]_i$ oscillations (periodicity: about 12 min) in renal epithelial cells, which stimulate the production of IL-6 and IL-8 in these cells. In addition, they observed that the calcium oscillations depend on the influx of $Ca^{2+}$ through voltage-operated L-type calcium channels in the plasma membrane, as well as on the release of $Ca^{2+}$ from stores within the ER through inositol trisphosphate receptor ($InsP_3R$) channels. A model based on these data suggests that interaction of HlyA with the cell membrane causes an influx of $Ca^{2+}$ through voltage-operated L-type calcium channels, and that an intracellular signal transduction pathway subsequently mediates activation of $InsP_3R$-gated $Ca^{2+}$ stores within the ER, resulting in the $[Ca^{2+}]_i$ oscillations (Söderblom *et al*., 2002). Although NF-κB activation by *E. coli* α-hemolysin has not yet been shown, it is likely that this transcription factor is involved in the α-hemolysin-induced IL-6 and IL-8 production in renal cells. In fact, Dolmetsch *et al*. (1998) demonstrated that expression of IL-8 is under control of artificially induced $Ca^{2+}$ oscillations due to frequency-modulated activation of NF-κB.

## CONCLUSION

A large quantity of new data regarding the structure and mode of action of RTX toxins has been compiled in the past years. Several things are, however, still poorly or incompletely understood. Examples are the mechanism of pore formation, the function of the fatty acylation and of the binding of calcium ions to RTX toxins, and the processes involved in the RTX toxin-mediated activation of host cells. Current work is aimed at further clarifying these issues.

## REFERENCES


Akatsuka, H., Kawai, E, Omori, K. and Shibatani, T. (1995). The three genes *lipB*, *lipC*, and *lipD* involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. *J. Bacteriol*. **177,** 6381–6389.

Andersen, C., Hughes, C. and Koronakis, V. (2002a). Electrophysiological behavior of the TolC channel-tunnel in planar lipid bilayers. *J. Membr. Biol*. **185,** 83–92.

Andersen, C., Koronakis, E., Bokma, E., Eswaran, J., Humphreys, D., Hughes, C. and Koronakis, V. (2002b). Transition to the open state of the TolC periplasmic tunnel entrance. *Proc. Natl. Acad. Sci. USA* **99,** 11103–11108.

Angelos, J.A., Hess, J.F. and George, L.W. (2003). An RTX operon in hemolytic *Moraxella bovis* is absent from nonhemolytic strains. *Vet. Microbiol*. **92,** 363–377.

Arnaout, M.A. (1990). Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* **75,** 1037–1050.

Awram, P. and Smit, J. (1998). The *Caulobacter crescentus* paracrystalline S-layer protein is secreted by an ABC transporter (type I) secretion apparatus. *J. Bacteriol*. **180,** 3062–3069.

Baeuerle, P.A. and Baltimore, D. (1996). NF-κB: Ten years after. *Cell* **87,** 13–20.

Bakas, L., Veiga, M.P., Soloaga, A., Ostolaza, H. and Goni, F.M. (1998). Calcium-dependent conformation of *E. coli* α-hemolysin. Implications for the mechanism of membrane insertion and lysis. *Biochim. Biophys. Acta* **1368,** 225–234.

Balakrishnan, L., Hughes, C. and Koronakis, V. (2001). Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by *Escherichia coli*. *J. Mol. Biol*. **313,** 501–510.

Barry, E.M., Weiss, A.A., Ehrmann, I.E., Gray, M.C., Hewlett, E.L. and Goodwin, M.St.M. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cyaC*, for activation. *J. Bacteriol*. **173,** 720–726.

Basar, T., Havlicek, V., Bezouskova, S., Hackett, M. and Sebo, P. (2001). Acylation of lysine 983 is sufficient for toxin activity of *Bordetella pertussis* adenylate cyclase. Substitutions of alanine 140 modulate acylation site selectivity of the toxin acyltransferase CyaC. *J. Biol. Chem*. **276,** 348–354.

Bassinet, L., Fitting, C., Housset, B., Cavaillon, J.-M. and Guiso, N. (2004). *Bordetella pertussis* adenylate cyclase-hemolysin induces interleukin-6 secretion by human tracheal epithelial cells. *Infect. Immun*. **72,** 5530–5533.

Bauer, M.E. and Welch, R.A. (1996a). Association of RTX toxins with erythrocytes. *Infect. Immun*. **64,** 4665–4672.

Bauer, M.E. and Welch, R.A. (1996b). Characterization of an RTX toxin from enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun*. **64,** 167–175.

Bauer, M.E. and Welch, R.A. (1997). Pleiotropic effects of a mutation in *rfaC* on *Escherichia coli* hemolysin. *Infect. Immun*. **65,** 2218–2224.

Baumann, U., Wu, S., Flaherty, K.M. and McKay, D.B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium binding parallel beta roll motif. *EMBO J*. **12,** 3357–3364.

Bejerano, M., Nisan, I., Ludwig, A., Goebel, W. and Hanski, E. (1999). Characterization of the C-terminal domain essential for toxic activity of adenylate cyclase toxin. *Mol. Microbiol*. **31,** 381–392.

Bellalou, J., Sakamoto, H., Ladant, D., Geoffroy, C. and Ullmann, A. (1990). Deletions affecting hemolytic and toxin activities of *Bordetella pertussis* adenylate cyclase. *Infect. Immun*. **58,** 3242–3247.

Benabdelhak, H., Kiontke, S., Horn, C., Ernst, R., Blight, M.A., Holland, I.B. and Schmitt, L. (2003). A specific interaction between the NBD of the ABC-transporter HlyB and a C-terminal fragment of its transport substrate haemolysin A. *J. Mol. Biol*. **327,** 1169–1179.

Benabdelhak, H., Schmitt, L., Horn, C., Jumel, K., Blight, M.A. and Holland, I.B. (2005). Positive co-operative activity and dimerization of the isolated ABC ATPase domain of HlyB from *Escherichia coli. Biochem. J*. **386,** 489–495.

Benz, R., Schmid, A., Wagner, W. and Goebel, W. (1989). Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. *Infect. Immun*. **57,** 887–895.

Benz, R., Hardie, K.R. and Hughes, C. (1994a). Pore formation in artificial membranes by the secreted hemolysins of *Proteus vulgaris* and *Morganella morganii. Eur. J. Biochem*. **220,** 339–347.

Benz, R., Maier, E., Ladant, D., Ullmann, A. and Sebo, P. (1994b). Adenylate cyclase toxin (CyaA) of *Bordetella pertussis*: evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli. J. Biol. Chem*. **269,** 27231–27239.

Berridge, M.J., Bootman, M.D. and Lipp, P. (1998). Calcium – a life and death signal. *Nature* **395,** 645–648.

Berthoud, H., Frey, J. and Kuhnert, P. (2002). Characterization of Aqx and its operon: the hemolytic RTX determinant of *Actinobacillus equuli. Vet. Microbiol*. **87,** 159–174.

Bhakdi, S. and Martin, E. (1991). Superoxide generation by human neutrophils induced by low doses of *Escherichia coli* hemolysin. *Infect. Immun*. **59,** 2955–2962.

Bhakdi, S., Mackman, N., Nicaud, J.-M. and Holland, I.B. (1986). *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infect. Immun*. **52,** 63–69.

Bhakdi, S., Greulich, S., Muhly, M., Eberspächer, B., Becker, H., Thiele, A. and Hugo, F. (1989). Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J. Exp. Med*. **169,** 737–754.

Bhakdi, S., Muhly, M., Korom, S. and Schmidt, G. (1990). Effects of *Escherichia coli* hemolysin on human monocytes. Cytocidal action and stimulation of interleukin 1 release. *J. Clin. Invest*. **85,** 1746–1753.

Boardman, B.K. and Fullner Satchell, K.J. (2004). *Vibrio cholerae* strains with mutations in an atypical type I secretion system accumulate RTX toxin intracellularly. *J. Bacteriol*. **186,** 8137–8143.

Boehm, D.F., Welch, R.A. and Snyder, I.S. (1990a). Calcium is required for binding of *Escherichia coli* hemolysin (HlyA) to erythrocyte membranes. *Infect. Immun*. **58,** 1951–1958.

Boehm, D.F., Welch, R.A. and Snyder, I.S. (1990b). Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. *Infect. Immun*. **58,** 1959–1964.

Bohach, G.A. and Snyder, I.S. (1985). Chemical and immunological analysis of the complex structure of *Escherichia coli* alpha-hemolysin. *J. Bacteriol*. **164,** 1071–1080.

Braun, M., Kuhnert, P., Nicolet, J., Burnens, A.P. and Frey, J. (1999). Cloning and characterization of two bistructural S-layer-RTX proteins from *Campylobacter rectus. J. Bacteriol*. **181,** 2501–2506.

Burrows, L.L. and Lo, R.Y.C. (1992). Molecular characterization of an RTX toxin determinant from *Actinobacillus suis. Infect. Immun*. **60,** 2166–2173.

Carbonetti, N.H., Artamonova, G.V., Andreasen, C. and Bushar, N. (2005). Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of *Bordetella pertussis* infection of the respiratory tract. *Infect. Immun*. **73,** 2698–2703.

Cavalieri, S.J. and Snyder, I.S. (1982). Effect of *Escherichia coli* α-hemolysin on human peripheral leukocyte function *in vitro*. *Infect. Immun*. **37,** 966–974.

Chang, Y.-F., Young, R. and Struck, D.K. (1989). Cloning and characterization of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae. DNA* **8,** 635–647.

Chang, Y.-F., Ma, D.-P., Shi, J. and Chengappa, M.M. (1993). Molecular characterization of a leukotoxin gene from a *Pasteurella haemolytica*-like organism, encoding a new member of the RTX toxin family. *Infect. Immun*. **61,** 2089–2095.

Chen, C.-Y., Wu, K.-M., Chang, Y.-C., Chang, C.-H., Tsai, H.-C., Liao, T.-L., Liu, Y.-M., Chen, H.-J., Shen, A.B.-T., Li, J.-C., Su, T.-L., Shao, C.-P., Lee, C.-T., Hor, L.-I. and Tsai, S.-F. (2003). Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res*. **13,** 2577–2587.

Chervaux, C. and Holland, I.B. (1996). Random and directed mutagenesis to elucidate the functional importance of helix II and F-989 in the C-terminal secretion signal of *Escherichia coli* hemolysin. *J. Bacteriol*. **178,** 1232–1236.

Clinkenbeard, K.D. and Upton, M.L. (1991). Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. *Am. J. Vet. Res*. **52,** 453–457.

Clinkenbeard, K.D., Mosier, D.A. and Confer, A.W. (1989a). Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **57,** 420–425.

Clinkenbeard, K.D., Mosier, D.A. and Confer, A.W. (1989b). Effects of *Pasteurella haemolytica* leukotoxin on isolated bovine neutrophils. *Toxicon* **27,** 797–804.

Clinkenbeard, K.D., Clarke, C.R., Hague, C.M., Clinkenbeard, P., Srikumaran, S. and Morton, R.J. (1994). *Pasteurella haemolytica* leukotoxin-induced synthesis of eicosanoids by bovine neutrophils *in vitro.* J. Leukoc. Biol. **56,** 644–649.

Confer, D.L. and Eaton, J.W. (1982). Phagocyte impotence caused by an invasive bacterial adenylate cyclase. *Science* **217,** 948–950.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2001). Glycophorin as a receptor for *Escherichia coli* α-haemolysin in erythrocytes. *J. Biol. Chem*. **276,** 12513–12519.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2003). A receptor-binding region in *Escherichia coli* α-haemolysin. *J. Biol. Chem*. **278,** 19159–19163.

Cruz, W.T., Young, R., Chang, Y.F. and Struck, D.K. (1990). Deletion analysis resolves cell-binding and lytic domains of the *Pasteurella* leukotoxin. *Mol. Microbiol*. **4,** 1933–1939.

Cudd, L., Clarke, C. and Clinkenbeard, K. (2003a). *Mannheimia haemolytica* leukotoxin-induced increase in leukotriene $B_4$ production by bovine neutrophils is mediated by a sustained and excessive increase in intracellular calcium concentration. *FEMS Microbiol. Lett*. **224,** 85–90.

Cudd, L., Clarke, C. and Clinkenbeard, K. (2003b). Contribution of intracellular calcium stores to an increase in cytosolic calcium concentration induced by *Mannheimia haemolytica* leukotoxin. *FEMS Microbiol. Lett*. **225,** 23–27.

Cullen, J.M. and Rycroft, A.N. (1994). Phagocytosis by pig alveolar macrophages of *Actinobacillus pleuropneumoniae* serotype 2 mutant strains defective in haemolysin II (ApxII) and pleurotoxin (ApxIII). *Microbiology*. **140,** 237–244.

Czuprynski, C.J. and Ortiz-Carranza, O. (1992). *Pasteurella haemolytica* leukotoxin inhibits mitogen-induced bovine peripheral blood mononuclear cell proliferation *in vitro. Microb. Pathog*. **12,** 459–463.

Czuprynski, C.J. and Welch, R.A. (1995). Biological effects of RTX toxins: the possible role of lipopolysaccharide. *Trends Microbiol*. **3,** 480–483.

Czuprynski, C.J., Noel, E.J., Ortiz-Carranza, O. and Srikumaran, S. (1991). Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **59,** 3126–3133.

Davies, R.L., Campbell, S. and Whittam, T.S. (2002). Mosaic structure and molecular evolution of the leukotoxin operon (*lktCABD*) in *Mannheimia* (*Pasteurella*) *haemolytica*, *Mannheimia glucosida*, and *Pasteurella trehalosi. J. Bacteriol*. **184,** 266–277.

Delepelaire, P. and Wandersman, C. (1990). Protein secretion in Gram-negative bacteria. The extracellular metalloprotease B from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that of *Escherichia coli* α-hemolysin. *J. Biol. Chem*. **265,** 17118–17125.

Demuth, D.R., James, D., Kowashi, Y. and Kato, S. (2003). Interaction of *Actinobacillus actinomycetemcomitans* outer membrane vesicles with HL60 cells does not require leukotoxin. *Cell. Microbiol*. **5,** 111–121.

Deshpande, M.S., Ambagala, T.C., Ambagala, A.P.N., Kehrli, M.E. Jr. and Srikumaran, S. (2002). Bovine CD18 is necessary and sufficient to mediate *Mannheimia* (*Pasteurella*) *haemolytica* leukotoxin-induced cytolysis. *Infect. Immun*. **70,** 5058–5064.

Dileepan, T., Thumbikat, P., Walcheck, B., Kannan, M.S. and Maheswaran, S.K. (2005). Recombinant expression of bovine LFA-1 and characterization of its role as a receptor for *Mannheimia haemolytica* leukotoxin. *Microb. Pathog*. **38,** 249–257.

Dinh, T., Paulsen, I.T. and Saier, M.H. (1994). A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. *J. Bacteriol*. **176,** 3825–3831.

Döbereiner, A., Schmid, A., Ludwig, A., Goebel, W. and Benz, R. (1996). The effects of calcium and other polyvalent cations on channel formation by *Escherichia coli* α-hemolysin in red blood cells and lipid bilayer membranes. *Eur. J. Biochem*. **240,** 454–460.

Dolmetsch, R.E., Xu, K. and Lewis, R.S. (1998). Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392,** 933–936.

D'Silva, P.R. and Lala, A.K. (2000). Organization of diphtheria toxin in membranes. A hydrophobic photolabeling study. *J. Biol. Chem*. **275,** 11771–11777.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.-F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin, A. and Kunst, F. (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens. Nat. Biotechnol*. **21,** 1307–1313.

Duong, F., Soscia, C., Lazdunski, A. and Murgier, M. (1994). The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. *Mol. Microbiol*. **11,** 1117–1126.

Duong, F., Lazdunski, A. and Murgier, M. (1996). Protein secretion by heterologous bacterial ABC-transporters: the C-terminus secretion signal of the secreted protein confers high recognition specificity. *Mol. Microbiol*. **21,** 459–470.

Eberspächer, B., Hugo, F. and Bhakdi, S. (1989). Quantitative study of the binding and hemolytic efficiency of *Escherichia coli* hemolysin. *Infect. Immun*. **57,** 983–988.

Economou, A., Hamilton, W.D.O., Johnston, A.W.B. and Downie, J.A. (1990). The *Rhizobium* nodulation gene *nodO* encodes a $Ca^{2+}$-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J*. **9,** 349–354.

Ehrmann, I.E., Weiss, A.A., Goodwin, M.S., Gray, M.C., Barry, E. and Hewlett, E.L. (1992). Enzymatic activity of adenylate cyclase toxin from *Bordetella pertussis* is not required for hemolysis. *FEBS Lett*. **304,** 51–56.

El-Azami-El-Idrissi, M., Bauche, C., Loucka, J., Osicka, R., Sebo, P., Ladant, D. and Leclerc, C. (2003). Interaction of *Bordetella pertussis* adenylate cyclase with CD11b/CD18: Role of toxin acylation and identification of the main integrin interaction domain. *J. Biol. Chem*. **278,** 38514–38521.

Eswaran, J., Hughes, C. and Koronakis, V. (2003). Locking TolC entrance helices to prevent protein translocation by the bacterial type I export apparatus. *J. Mol. Biol*. **327,** 309–315.

Felmlee, T., Pellett, S. and Welch, R.A. (1985). Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol*. **163,** 94–105.

Forestier, C. and Welch, R.A. (1990). Nonreciprocal complementation of the *hlyC* and *lktC* genes of the *Escherichia coli* hemolysin and *Pasteurella haemolytica* leukotoxin determinants. *Infect. Immun*. **58,** 828–832.

Frey, J. (1995). Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. *Trends Microbiol*. **3,** 257–261.

Frey, J., Meier, R., Gygi, D. and Nicolet, J. (1991). Nucleotide sequence of the hemolysin I gene from *Actinobacillus pleuropneumoniae. Infect. Immun*. **59,** 3026–3032.

Frey, J., van den Bosch, H., Segers, R. and Nicolet, J. (1992). Identification of a second hemolysin (HlyII) in *Actinobacillus pleuropneumoniae* serotype 1 and expression of the gene in *Escherichia coli. Infect. Immun*. **60,** 1671–1676.

Frey, J., Beck, M., Stucki, U. and Nicolet, J. (1993a). Analysis of hemolysin operons in *Actinobacillus pleuropneumoniae. Gene* **123,** 51–58.

Frey, J., Bosse, J.T., Chang, Y.-F., Cullen, J.M., Fenwick, B., Gerlach, G.F., Gygi, D., Haesebrouck, F., Inzana, T.J., Jansen, R., Kamp, E.M., Macdonald, J., MacInnes, J.I., Mittal, K.R., Nicolet, J., Rycroft, A.N., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Struck, D.K., van den Bosch, J.F., Willson, P.J. and Young, R. (1993b). *Actinobacillus pleuropneumoniae* RTX-toxins: uniform designation of haemolysins, cytolysins, pleurotoxin and their genes. *J. Gen. Microbiol*. **139,** 1723–1728.

Friedman, R.L., Fiederlein, R.L., Glasser, L. and Galgiani, J.N. (1987). *Bordetella pertussis* adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. *Infect. Immun*. **55,** 135–140.

Fullner, K.J. and Mekalanos, J.J. (2000). *In vivo* covalent cross-linking of cellular actin by the *Vibrio cholerae* RTX toxin. *EMBO J*. **19,** 5315–5323.

Fullner, K.J., Lencer, W.I. and Mekalanos, J.J. (2001). *Vibrio cholerae*-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. *Infect. Immun*. **69,** 6310–6317.

Fullner, K.J., Boucher, J.C., Hanes, M.A., Haines, G.K. 3rd, Meehan, B.M., Walchle, C., Sansonetti, P.J. and Mekalanos, J.J. (2002). The contribution of accessory toxins of *Vibrio cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. *J. Exp. Med*. **195,** 1455–1462.

Gangola, P. and Rosen, B.P. (1987). Maintenance of intracellular calcium in *Escherichia coli. J. Biol. Chem*. **262,** 12570–12574.

Gentschev, I. and Goebel, W. (1992). Topological and functional studies on HlyB of *Escherichia coli. Mol. Gen. Genet*. **232,** 40–48.

Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A. and Danchin, A. (1988). Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis. EMBO J*. **7,** 3997–4004.

Goebel, W. and Hedgpeth, J. (1982). Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli. J. Bacteriol*. **151,** 1290–1298.

Goodwin, M.St.M. and Weiss, A.A. (1990). Adenylate cyclase toxin is critical for colonization and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infect. Immun*. **58,** 3445–3447.

Gray, L., Mackman, N., Nicaud, J.-M. and Holland, I.B. (1986). The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from *Escherichia coli. Mol. Gen. Genet*. **205,** 127–133.

Gray, M., Szabo, G., Otero, A.S., Gray, L. and Hewlett, E. (1998). Distinct mechanisms for $K^+$ efflux, intoxication, and hemolysis by *Bordetella pertussis* AC toxin. *J. Biol. Chem*. **273,** 18260–18267.

Gray, M.C., Ross, W., Kim, K. and Hewlett, E.L. (1999). Characterization of binding of adenylate cyclase toxin to target cells by flow cytometry. *Infect. Immun*. **67,** 4393–4399.

Gray, M.C., Lee, S.-J., Gray, L.S., Zaretzky, F.R., Otero, A.S., Szabo, G. and Hewlett, E.L. (2001). Translocation-specific conformation of adenylate cyclase toxin from *Bordetella pertussis* inhibits toxin-mediated hemolysis. *J. Bacteriol*. **183,** 5904–5910.

Gray, M.C., Donato, G.M., Jones, F.R., Kim, T. and Hewlett, E.L. (2004). Newly secreted adenylate cyclase toxin is responsible for intoxication of target cells by *Bordetella pertussis. Mol. Microbiol*. **53,** 1709–1719.

Grimminger, F., Scholz, C., Bhakdi, S. and Seeger, W. (1991a). Subhemolytic doses of *Escherichia coli* hemolysin evoke large quantities of lipoxygenase products in human neutrophils. *J. Biol. Chem*. **266,** 14262–14269.

Grimminger, F., Sibelius, U., Bhakdi, S., Suttorp, N. and Seeger, W. (1991b). *Escherichia coli* hemolysin is a potent inductor of phosphoinositide hydrolysis and related metabolic responses in human neutrophils. *J. Clin. Invest*. **88,** 1531–1539.

Grimminger, F., Rose, F., Sibelius, U., Meinhardt, M., Pötzsch, B., Spriestersbach, R., Bhakdi, S., Suttorp, N. and Seeger, W. (1997). Human endothelial cell activation and mediator release in response to the bacterial exotoxins *Escherichia coli* hemolysin and Staphylococcal α-toxin. *J. Immunol*. **159,** 1909–1916.

Gross, M.K., Au, D.C., Smith, A.L. and Storm, D.R. (1992). Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional cyaA toxin of *Bordetella pertussis* abolish virulence. *Proc. Natl. Acad. Sci. USA* **89,** 4898–4902.

Gueirard, P., Druilhe, A., Pretolani, M. and Guiso, N. (1998). Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection *in vivo. Infect. Immun*. **66,** 1718–1725.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the $\alpha_M\beta_2$ integrin (CD11b/CD18). *J. Exp. Med*. **193,** 1035–1044.

Guha, M. and Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cell. Signal*. **13,** 85–94.

Hacker, J., Hughes, C., Hof, H. and Goebel, W. (1983). Cloned hemolysin genes from *Escherichia coli* that cause urinary tract infection determine different levels of toxicity in mice. *Infect. Immun*. **42,** 57–63.

Hackett, M., Guo, L., Shabanowitz, J., Hunt, D.F. and Hewlett, E.L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis. Science* **266,** 433–435.

Hackett, M., Walker, C.B., Guo, L., Gray, M.C., Van Cuyk, S., Ullmann, A., Shabanowitz, J., Hunt, D.F., Hewlett, E.L. and Sebo, P. (1995). Hemolytic, but not cell-invasive activity, of adenylate cyclase toxin is selectively affected by differential fatty-acylation in *Escherichia coli. J. Biol. Chem*. **270,** 20250–20253.

Haraszthy, V.I., Hariharan, G., Tinoco, E.M.B., Cortelli, J.R., Lally, E.T., Davis, E. and Zambon, J.J. (2000). Evidence for the role of highly leukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. *J. Periodontol*. **71,** 912–922.

Havlicek, V., Higgins, L., Chen, W., Halada, P., Sebo, P., Sakamoto, H. and Hackett, M. (2001). Mass spectrometric analysis of recombinant adenylate cyclase toxin from *Bordetella pertussis* strain 18323/pHSP9. *J. Mass Spectrom*. **36,** 384–391.

Henricks, P.A.J., Binkhorst, G.J., Drijver, A.A. and Nijkamp, F.P. (1992). *Pasteurella haemolytica* leukotoxin enhances production of leukotriene $B_4$ and 5-hydroxyeicosatetraenoic acid by bovine polymorphonuclear leukocytes. *Infect. Immun*. **60,** 3238–3243.

Herlax, V., de Alaniz, M.J. and Bakas, L. (2005). Role of lipopolysaccharide on the structure and function of α-hemolysin from *Escherichia coli. Chem. Phys. Lipids* **135,** 107–115.

Hess, J., Wels, W., Vogel, M. and Goebel, W. (1986). Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. *FEMS Microbiol. Lett*. **34,** 1–11.

Hess, J., Gentschev, I., Goebel, W. and Jarchau, T. (1990). Analysis of the haemolysin secretion system by PhoA-HlyA fusion proteins. *Mol. Gen. Genet*. **224,** 201–208.

Hewlett, E.L., Urban, M.A., Manclark, C.R. and Wolff, J. (1976). Extracytoplasmic adenylate cyclase of *Bordetella pertussis. Proc. Natl. Acad. Sci. USA* **73,** 1926–1930.

Hewlett, E.L., Gordon, V.M., McCaffery, J.D., Sutherland, W.M. and Gray, M.C. (1989). Adenylate cyclase toxin from *Bordetella pertussis*: identification and purification of the holotoxin molecule. *J. Biol. Chem*. **264,** 19379–19384.

Hewlett, E.L., Gray, L., Allietta, M., Ehrmann, I., Gordon, V.M. and Gray, M.C. (1991). Adenylate cyclase toxin from *Bordetella pertussis*: conformational change associated with toxin activity. *J.Biol. Chem*. **266,** 17503–17508.

Hewlett, E.L., Gray, M.C., Ehrmann, I.E., Maloney, N.J., Otero, A.S., Gray, L., Allietta, M., Szabo, G., Weiss, A.A. and Barry, E.M. (1993). Characterization of adenylate cyclase toxin from a mutant of *Bordetella pertussis* defective in the activator gene, *cyaC. J. Biol. Chem*. **268,** 7842–7848.

Higgins, C.F. (1992). ABC transporters: from microorganisms to man. *Annu. Rev. Cell. Biol*. **8,** 67–113.

Highlander, S.K., Fedorova, N.D., Dusek, D.M., Panciera, R., Alvarez, L.E. and Rinehart, C. (2000). Inactivation of *Pasteurella* (*Mannheimia*) *haemolytica* leukotoxin causes partial attenuation of virulence in a calf challenge model. *Infect. Immun*. **68,** 3916–3922.

Hormozi, K., Parton, R. and Coote, J. (1998). Target cell specificity of the *Pasteurella haemolytica* leukotoxin is unaffected by the nature of the fatty-acyl group used to activate the toxin *in vitro. FEMS Microbiol. Lett*. **169,** 139–145.

Hritz, M., Fisher, E. and Demuth, D.R. (1996). Differential regulation of the leukotoxin operon in highly leukotoxic and minimally leukotoxic strains *of Actinobacillus actinomycetemcomitans. Infect. Immun*. **64,** 2724–2729.

Hsuan, S.L., Kannan, M.S., Jeyaseelan, S., Prakash, Y.S., Sieck, G.C. and Maheswaran, S.K. (1998). *Pasteurella haemolytica* A1-derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. *Infect. Immun*. **66,** 2836–2844.

Hsuan, S.L., Kannan, M.S., Jeyaseelan, S., Prakash, Y.S., Malazdrewich, C., Abrahamsen, M.S., Sieck, G.C. and Maheswaran, S.K. (1999). *Pasteurella haemolytica* leukotoxin and endotoxin-induced cytokine gene expression in bovine alveolar macrophages requires NF-κB activation and calcium elevation. *Microb. Pathog*. **26,** 263–273.

Hughes, H.P.A., Campos, M., McDougall, L., Beskorwayne, T.K., Potter, A.A. and Babiuk, L.A. (1994). Regulation of major histocompatibility complex class II expression by *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **62,** 1609–1615.

Hui, D. and Ling, V. (2002). A combinatorial approach toward analyzing functional elements of the *Escherichia coli* hemolysin signal sequence. *Biochemistry* **41,** 5333–5339.

Hui, D., Morden, C., Zhang, F. and Ling, V. (2000). Combinatorial analysis of the structural requirements of the *Escherichia coli* hemolysin signal sequence. *J. Biol. Chem*. **275,** 2713–2720.

Hyland, C., Vuillard, L., Hughes, C. and Koronakis, V. (2001). Membrane interaction of *Escherichia coli* hemolysin: flotation and insertion-dependent labeling by phospholipid vesicles. *J. Bacteriol*. **183,** 5364–5370.

Issartel, J.-P., Koronakis, V. and Hughes, C. (1991). Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* **351,** 759–761.

Iwaki, M., Ullmann, A. and Sebo, P. (1995). Identification by *in vitro* complementation of regions required for cell-invasive activity of *Bordetella pertussis* adenylate cyclase toxin. *Mol. Microbiol*. **17,** 1015–1024.

Jansen, R., Briaire, J., Kamp, E.M. and Smits, M.A. (1992). Comparison of the cytolysin II genetic determinants of *Actinobacillus pleuropneumoniae* serotypes. *Infect. Immun*. **60,** 630–636.

Jansen, R., Briaire, J., Kamp, E.M., Gielkens, A.L.J. and Smits, M.A. (1993a). Cloning and characterization of the *Actinobacillus pleuropneumoniae*-RTX-toxin III (ApxIII) gene. *Infect. Immun*. **61,** 947–954.

Jansen, R., Briaire, J., Kamp, E.M., Gielkens, A.L.J. and Smits, M.A. (1993b). Structural analysis of the *Actinobacillus pleuropneumoniae*-RTX-toxin I (ApxI) operon. *Infect. Immun*. **61,** 3688–3695.

Jansen, R., Briaire, J., van Geel, A.B.M., Kamp, E.M., Gielkens, A.L.J. and Smits, M.A. (1994). Genetic map of the *Actinobacillus pleuropneumoniae* RTX-toxin (Apx) operons: characterization of the ApxIII operons. *Infect. Immun*. **62,** 4411–4418.

Jansen, R., Briaire, J., Smith, H.E., Dom, P., Haesebrouck, F., Kamp, E.M., Gielkens, A.L.J. and Smits, M.A. (1995). Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. *Infect. Immun*. **63,** 27–37.

Jarchau, T., Chakraborty, T., Garcia, F. and Goebel, W. (1994). Selection for transport competence of C-terminal polypeptides derived from *Escherichia coli* hemolysin: the shortest peptide capable of autonomous HlyB/HlyD-dependent secretion comprises the C-terminal 62 amino acids of HlyA. *Mol. Gen. Genet*. **245,** 53–60.

Jeyaseelan, S., Hsuan, S.L., Kannan, M.S., Walcheck, B., Wang, J.F., Kehrli, M.E., Lally, E.T., Sieck, G.C. and Maheswaran, S.K. (2000). Lymphocyte function-associated antigen 1 is a receptor for *Pasteurella haemolytica* leukotoxin in bovine leukocytes. *Infect. Immun*. **68,** 72–79.

Johansson, A., Claesson, R., Hänström, L., Sandström, G. and Kalfas, S. (2000). Polymorphonuclear leukocyte degranulation induced by leukotoxin from *Actinobacillus actinomycetemcomitans. J. Periodontal Res*. **35,** 85–92.

Johnson, J.M. and Church, G.M. (1999). Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J. Mol. Biol*. **287,** 695–715.

Jonas, D., Schultheis, B., Klas, C., Krammer, P.H. and Bhakdi, S. (1993). Cytocidal effects of *Escherichia coli* hemolysin on human T lymphocytes. *Infect. Immun*. **61,** 1715–1721.

Jorgensen, S.E., Mulcahy, P.F., Wu, G.K. and Louis, C.F. (1983). Calcium accumulation in human and sheep erythrocytes that is induced by *Escherichia coli* hemolysin. *Toxicon* **21,** 717–727.

Kachlany, S.C., Fine, D.H. and Figurski, D.H. (2000). Secretion of RTX leukotoxin by *Actinobacillus actinomycetemcomitans. Infect. Immun*. **68,** 6094–6100.

Kaehler, K.L., Markham, R.J., Muscoplat, C.C. and Johnson, D.W. (1980). Evidence of species specificity in the cytocidal effects of *Pasteurella haemolytica*. *Infect. Immun*. **30,** 615–616.

Kamp, E.M., Popma, J.K., Anakotta, J. and Smits, M.A. (1991). Identification of hemolytic and cytotoxic proteins of *Actinobacillus pleuropneumoniae* by use of monoclonal antibodies. *Infect. Immun*. **59,** 3079–3085.

Kamp, E.M., Vermeulen, T.M.M., Smits, M.A. and Haagsma, J. (1994). Production of Apx toxins by field strains of *Actinobacillus pleuropneumoniae* and *Actinobacillus suis. Infect. Immun*. **62,** 4063–4065.

Kamp, E.M., Stockhofe-Zurwieden, N., van Leengoed, L.A. and Smits, M.A. (1997). Endobronchial inoculation with Apx toxins of *Actinobacillus pleuropneumoniae* leads to pleuropneumonia in pigs. *Infect. Immun*. **65,** 4350–4354.

Karakelian, D., Lear, J.D., Lally, E.T. and Tanaka, J.C. (1998). Characterization of *Actinobacillus actinomycetemcomitans* leukotoxin pore formation in HL60 cells. *Biochim. Biophys. Acta* **1406,** 175–187.

Kato, S., Kowashi, Y. and Demuth, D.R. (2002). Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microb. Pathog*. **32,** 1–13.

Kawai, E., Akatsuka, H., Idei, A., Shibatani, T. and Omori, K. (1998). *Serratia marcescens* S-layer protein is secreted extracellularly via an ATP-binding cassette exporter, the Lip system. *Mol. Microbiol*. **27,** 941–952.

Kelk, P., Johansson, A., Claesson, R., Hänström, L. and Kalfas, S. (2003). Caspase 1 involvement in human monocyte lysis induced by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infect. Immun*. **71,** 4448–4455.

Kelk, P., Claesson, R., Hänström, L., Lerner, U.H., Kalfas, S. and Johansson, A. (2005). Abundant secretion of bioactive interleukin-1β by human macrophages induced by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infect. Immun*. **73,** 453–458.

Kenny, B., Chervaux, C. and Holland, I.B. (1994). Evidence that residues -15 to -46 of the haemolysin secretion signal are involved in early steps in secretion, leading to recognition of the translocator. *Mol. Microbiol*. **11,** 99–109.

Khelef, N. and Guiso, N. (1995). Induction of macrophage apoptosis by *Bordetella pertussis* adenylate cyclase-hemolysin. *FEMS Microbiol. Lett*. **134,** 27–32.

Khelef, N., Bachelet, C.-M., Vargaftig, B.B. and Guiso, N. (1994). Characterization of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesins or toxins. *Infect. Immun*. **62,** 2893–2900.

Khelef, N., Gounon, P. and Guiso, N. (2001). Internalization of *Bordetella pertussis* adenylate cyclase-haemolysin into endocytic vesicles contributes to macrophage cytotoxicity. *Cell. Microbiol*. **3,** 721–730.

Kolodrubetz, D., Spitznagel, J., Wang, B., Phillips, L.H., Jacobs, C. and Kraig, E. (1996). *cis* elements and *trans* factors are both important in strain-specific regulation of the leukotoxin gene in *Actinobacillus actinomycetemcomitans. Infect. Immun*. **64,** 3451–3460.

König, B., König, W., Scheffer, J., Hacker, J. and Goebel, W. (1986). Role of *Escherichia coli* alpha-hemolysin and bacterial adherence in infection: requirement for release of inflammatory mediators from granulocytes and mast cells. *Infect. Immun*. **54,** 886–892.

König, B., Schönfeld, W., Scheffer, J. and König, W. (1990). Signal transduction in human platelets and inflammatory mediator release induced by genetically cloned hemolysin-positive and -negative *Escherichia coli* strains. *Infect. Immun*. **58,** 1591–1599.

Koronakis, E., Hughes, C., Milisav, I. and Koronakis, V. (1995). Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol. Microbiol*. **16,** 87–96.

Koronakis, V., Cross, M., Senior, B., Koronakis, E. and Hughes, C. (1987). The secreted hemolysins of *Proteus mirabilis, Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. *J. Bacteriol*. **169,** 1509–1515.

Koronakis, V., Koronakis, E. and Hughes, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO J*. **8,** 595–605.

Koronakis, V., Hughes, C. and Koronakis, E. (1991). Energetically distinct early and late stages of HlyB/HlyD-dependent secretion across both *Escherichia coli* membranes. *EMBO J*. **10,** 3263–3272.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. and Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405,** 914–919.

Koronakis, V., Eswaran, J. and Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. *Annu. Rev. Biochem*. **73,** 467–489.

Korostoff, J., Wang, J.F., Kieba, I., Miller, M., Shenker, B.J. and Lally, E.T. (1998). *Actinobacillus actinomycetemcomitans* leukotoxin induces apoptosis in HL-60 cells. *Infect. Immun*. **66,** 4474–4483.

Kraig, E., Dailey, T. and Kolodrubetz, D. (1990). Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. *Infect. Immun*. **58,** 920–929.

Kuhnert, P., Heyberger-Meyer, B., Nicolet, J. and Frey, J. (2000). Characterization of PaxA and its operon: a cohemolytic RTX toxin determinant from pathogenic *Pasteurella aerogenes*. *Infect. Immun*. **68,** 6–12.

Kuhnert, P., Berthoud, H., Straub, R. and Frey, J. (2003). Host cell specific activity of RTX toxins from haemolytic *Actinobacillus equuli* and *Actinobacillus suis*. *Vet. Microbiol*. **92,** 161–167.

Kuhnert, P., Schlatter, Y. and Frey, J. (2005). Characterization of the type I secretion system of the RTX toxin ApxII in "*Actinobacillus porcitonsillarum*". *Vet. Microbiol*. **107,** 225–232.

Lafleur, R.L., Malazdrewich, C., Jeyaseelan, S., Bleifield, E., Abrahamsen, M.S. and Maheswaran, S.K. (2001). Lipopolysaccharide enhances cytolysis and inflammatory cytokine induction in bovine alveolar macrophages exposed to *Pasteurella* (*Mannheimia*) *haemolytica* leukotoxin. *Microb. Pathog*. **30,** 347–357.

Lally, E.T., Golub, E.E., Kieba, I.R., Taichman, N.S., Rosenbloom, J., Rosenbloom, J.C., Gibson, C.W. and Demuth, D.R. (1989). Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene. Delineation of unique features and comparison to homologous toxins. *J. Biol. Chem*. **264,** 15451–15456.

Lally, E.T., Kieba, I.R., Sato, A., Green, C.L., Rosenbloom, J., Korostoff, J., Wang, J.F., Shenker, B.J., Ortlepp, S., Robinson, M.K. and Billings, P.C. (1997). RTX toxins recognize a β2 integrin on the surface of human target cells. *J. Biol. Chem*. **272,** 30463–30469.

Langston, K.G., Worsham, L.M.S., Earls, L. and Ernst-Fonberg, M.L. (2004). Activation of hemolysin toxin: relationship between two internal protein sites of acylation. *Biochemistry* **43,** 4338–4346.

Lear, J.D., Furblur, U.G., Lally, E.T. and Tanaka, J.C. (1995). *Actinobacillus actinomycetemcomitans* leukotoxin forms large conductance, voltage-gated ion channels when incorporated into planar lipid bilayers. *Biochim. Biophys. Acta* **1238,** 34–41.

Lee, S.-J., Gray, M.C., Zu, K. and Hewlett, E.L. (2005). Oligomeric behavior of *Bordetella pertussis* adenylate cyclase toxin in solution. *Arch. Biochem. Biophys*. **438,** 80–87.

Leite, F., Brown, J.F., Sylte, M.J., Briggs, R.E. and Czuprynski, C.J. (2000). Recombinant bovine interleukin-1β amplifies the effects of partially purified *Pasteurella haemolytica* leukotoxin on bovine neutrophils in a $\beta_2$-integrin-dependent manner. *Infect. Immun*. **68,** 5581–5586.

Leite, F., O'Brien, S., Sylte, M.J., Page, T., Atapattu, D. and Czuprynski, C.J. (2002). Inflammatory cytokines enhance the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral blood neutrophils *in vitro*. *Infect. Immun*. **70,** 4336–4343.

Leite, F., Gyles, S., Atapattu, D., Maheswaran, S.K. and Czuprynski, C.J. (2003). Prior exposure to *Mannheimia haemolytica* leukotoxin or LPS enhances $\beta_2$-integrin expression by bovine neutrophils and augments LKT cytotoxicity. *Microb. Pathog*. **34,** 267–275.

Leite, F., Kuckleburg, C., Atapattu, D., Schultz, R. and Czuprynski, C.J. (2004). BHV-1 infection and inflammatory cytokines amplify the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral blood mononuclear cells in vitro. *Vet. Immunol. Immunopathol*. **99,** 193–202.

Leite, F., Atapattu, D., Kuckleburg, C., Schultz, R. and Czuprynski, C.J. (2005). Incubation of bovine PMNs with conditioned medium from BHV-1 infected peripheral blood mononuclear cells increases their susceptibility to *Mannheimia haemolytica* leukotoxin. *Vet. Immunol. Immunopathol*. **103,** 187–193.

Lesieur, C., Vecsey-Semjen, B., Abrami, L., Fivaz, M. and van der Goot, F.G. (1997). Membrane insertion: the strategies of toxins. *Mol. Membr. Biol*. **14,** 45–64.

Letoffe, S., Delepelaire, P. and Wandersman, C. (1991). Cloning and expression in *Escherichia coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. *J. Bacteriol*. **173,** 2160–2166.

Li, J. and Clinkenbeard, K.D. (1999). Lipopolysaccharide complexes with *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **67,** 2920–2927.

Li, J., Clinkenbeard, K.D. and Ritchey, J.W. (1999). Bovine CD18 identified as a species specific receptor for *Pasteurella haemolytica* leukotoxin. *Vet. Microbiol*. **67,** 91–97.

Lilie, H., Haehnel, W., Rudolph, R. and Baumann, U. (2000). Folding of a synthetic parallel β-roll protein. *FEBS Lett*. **470,** 173–177.

Lim, K.B., Bazemore Walker, C.R., Guo, L., Pellett, S., Shabanowitz, J., Hunt, D.F., Hewlett, E.L., Ludwig, A., Goebel, W., Welch, R.A. and Hackett, M. (2000). *Escherichia coli* α-hemolysin (HlyA) is heterogeneously acylated *in vivo* with 14-, 15-, and 17-carbon fatty acids. *J. Biol. Chem*. **275,** 36698–36702.

Lin, W., Fullner, K.J., Clayton, R., Sexton, J.A., Rogers, M.B., Calia, K.E., Calderwood, S.B., Fraser, C. and Mekalanos, J.J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc. Nat. Acad. Sci. USA* **96,** 1071–1076.

Lo, R.Y.C., Strathdee, C.A. and Shewen, P.E. (1987). Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infect. Immun*. **55,** 1987–1996.

Ludwig, A., Vogel, M. and Goebel, W. (1987). Mutations affecting activity and transport of haemolysin in *Escherichia coli*. *Mol. Gen. Genet*. **206,** 238–245.

Ludwig, A., Jarchau, T., Benz, R. and Goebel, W. (1988). The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its $Ca^{2+}$-dependent binding to erythrocytes. *Mol. Gen. Genet*. **214,** 553–561.

Ludwig, A., Schmid, A., Benz, R. and Goebel, W. (1991). Mutations affecting pore formation by haemolysin from *Escherichia coli*. *Mol. Gen. Genet*. **226,** 198–208.

Ludwig, A., Benz, R. and Goebel, W. (1993). Oligomerization of *Escherichia coli* haemolysin (HlyA) is involved in pore formation. *Mol. Gen. Genet*. **241,** 89–96.

Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J. and Goebel, W. (1996). Analysis of the *in vivo* activation of hemolysin (HlyA) from *Escherichia coli*. *J. Bacteriol*. **178,** 5422–5430.

Macdonald, J. and Rycroft, A.N. (1993). *Actinobacillus pleuropneumoniae* haemolysin II is secreted from *Escherichia coli* by *A. pleuropneumoniae* pleurotoxin secretion gene products. *FEMS Microbiol. Lett*. **109,** 317–322.

Maheswaran, S.K., Kannan, M.S., Weiss, D.J., Reddy, K.R., Townsend, E.L., Yoo, H.S., Lee, B.W. and Whiteley, L.O. (1993). Enhancement of neutrophil-mediated injury to bovine pulmonary endothelial cells by *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **61,** 2618–2625.

Maier, E., Reinhard, N., Benz, R. and Frey, J. (1996). Channel-forming activity and channel size of the RTX toxins ApxI, ApxII, and ApxIII of *Actinobacillus pleuropneumoniae*. *Infect. Immun*. **64,** 4415–4423.

Mangan, D.F., Taichman, N.S., Lally, E.T. and Wahl, S.M. (1991). Lethal effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. *Infect. Immun*. **59,** 3267–3272.

Martin, C., Requero, M.-A., Masin, J., Konopasek, I., Goni, F.M., Sebo, P. and Ostolaza, H. (2004). Membrane restructuring by *Bordetella pertussis* adenylate cyclase toxin, a member of the RTX toxin family. *J. Bacteriol*. **186,** 3760–3765.

May, A.K., Sawyer, R.G., Gleason, T., Whitworth, A. and Pruett, T.L. (1996). *In vivo* cytokine response to *Escherichia coli* alpha-hemolysin determined with genetically engineered hemolytic and nonhemolytic *E. coli* variants. *Infect. Immun*. **64,** 2167–2171.

May, A.K., Gleason, T.G., Sawyer, R.G. and Pruett, T.L. (2000). Contribution of *Escherichia coli* alpha-hemolysin to bacterial virulence and to intraperitoneal alterations in peritonitis. *Infect. Immun*. **68,** 176–183.

Menestrina, G., Mackman, N., Holland, I.B. and Bhakdi, S. (1987). *Escherichia coli* haemolysin forms voltage-dependent ion channels in lipid membranes. *Biochim. Biophys. Acta* **905,** 109–117.

Menestrina, G., Pederzolli, C., Dalla Serra, M., Bregante, M. and Gambale, F. (1996). Permeability increase induced by *Escherichia coli* hemolysin A in human macrophages is due to the formation of ionic pores: a patch clamp characterization. *J. Membrane Biol*. **149,** 113–121.

Moayeri, M. and Welch, R.A. (1994). Effects of temperature, time, and toxin concentration on lesion formation by the *Escherichia coli* hemolysin. *Infect. Immun*. **62,** 4124–4134.

Moayeri, M. and Welch, R.A. (1997). Prelytic and lytic conformations of erythrocyte-associated *Escherichia coli* hemolysin. *Infect. Immun*. **65,** 2233–2239.

Mobley, H.L.T., Green, D.M., Trifillis, A.L., Johnson, D.E., Chippendale, G.R., Lockatell, C.V., Jones, B.D. and Warren, J.W. (1990). Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. *Infect. Immun*. **58,** 1281–1289.

Murphy, G.L., Whitworth, L.C., Clinkenbeard, K.D. and Clinkenbeard, P.A. (1995). Hemolytic activity of the *Pasteurella haemolytica* leukotoxin. *Infect. Immun*. **63,** 3209–3212.

O'Hanley, P., Lalonde, G. and Ji, G. (1991). Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding *Escherichia coli* in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis. *Infect. Immun*. **59,** 1153–1161.

Ohta, H., Hara, H., Fukui, K., Kurihara, H., Murayama, Y. and Kato, K. (1993). Association of *Actinobacillus actinomycetemcomitans* leukotoxin with nucleic acids on the bacterial cell surface. *Infect. Immun*. **61,** 4878–4884.

Oropeza-Wekerle, R.-L., Speth, W., Imhof, B., Gentschev, I. and Goebel, W. (1990). Translocation and compartmentalization of *Escherichia coli* haemolysin (HlyA). *J. Bacteriol*. **172,** 3711–3717.

Oropeza-Wekerle, R.-L., Muller, S., Briand, J.-P., Benz, R., Schmid, A. and Goebel, W. (1992). Haemolysin-derived synthetic peptides with pore-forming and haemolytic activity. *Mol. Microbiol*. **6,** 115–121.

Ortiz-Carranza, O. and Czuprynski, C.J. (1992). Activation of bovine neutrophils by *Pasteurella haemolytica* leukotoxin is calcium dependent. *J. Leukoc. Biol*. **52,** 558–564.

Osickova, A., Osicka, R. Maier, E., Benz, R. and Sebo, P. (1999). An amphipathic α-helix including glutamates 509 and 516 is crucial for membrane translocation of adenylate cyclase toxin and modulates formation and cation selectivity of its membrane channels. *J. Biol. Chem*. **274,** 37644–37650.

Ostolaza, H., Soloaga, A. and Goni, F.M. (1995). The binding of divalent cations to *Escherichia coli* α-haemolysin. *Eur. J. Biochem*. **228,** 39–44.

Otero, A.S., Yi, X.B., Gray, M.C., Szabo, G. and Hewlett, E.L. (1995). Membrane depolarization prevents cell invasion by *Bordetella pertussis* adenylate cyclase toxin. *J. Biol. Chem*. **270,** 9695–9697.

Pearson, R.D., Symes, P., Conboy, M., Weiss, A.A. and Hewlett, E.L. (1987). Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. *J. Immunol*. **139,** 2749–2754.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Petras, S.F., Chidambaram, M., Illyes, E.F., Froshauer, S., Weinstock, G.M. and Reese, C.P. (1995). Antigenic and virulence properties of *Pasteurella haemolytica* leukotoxin mutants. *Infect. Immun*. **63,** 1033–1039.

Pimenta, A.L., Young, J., Holland, I.B. and Blight, M.A. (1999). Antibody analysis of the localisation, expression and stability of HlyD, the MFP component of the *E. coli* haemolysin translocator. *Mol. Gen. Genet*. **261,** 122–132.

Rhodes, C.R., Gray, M.C., Watson, J.M., Muratore, T.L., Kim, S.B., Hewlett, E.L. and Grisham, C.M. (2001). Structural consequences of divalent metal binding by the adenylyl cyclase toxin of *Bordetella pertussis*. *Arch. Biochem. Biophys*. **395,** 169–176.

Rogel, A. and Hanski, E. (1992). Distinct steps in the penetration of adenylate cyclase toxin of *Bordetella pertussis* into sheep erythrocytes: translocation of the toxin across the membrane. *J. Biol. Chem*. **267,** 22599–22605.

Rogel, A., Schultz, J.E., Brownlie, R.M., Coote, J.G., Parton, R. and Hanski, E. (1989). *Bordetella pertussis* adenylate cyclase: purification and characterization of the toxic form of the enzyme. *EMBO J*. **8,** 2755–2760.

Rogel, A., Meller, R. and Hanski, E. (1991). Adenylate cyclase toxin from *Bordetella pertussis*: the relationship between induction of cAMP and hemolysis. *J. Biol. Chem*. **266,** 3154–3161.

Rose, T., Sebo, P., Bellalou, J. and Ladant, D. (1995). Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin. Characterization of multiple calcium-binding sites and calcium-induced conformational changes. *J. Biol. Chem*. **270,** 26370–26376.

Ross, P.J., Lavelle, E.C., Mills, K.H.G. and Boyd, A.P. (2004). Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infect. Immun*. **72,** 1568–1579.

Sakamoto, H., Bellalou, J., Sebo, P. and Ladant, D. (1992). *Bordetella pertussis* adenylate cyclase toxin: structural and functional independence of the catalytic and hemolytic activities. *J. Biol. Chem*. **267,** 13598–13602.

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., MacInnes, J.I., Segers, R.P.A.M. and Frey, J. (1999). Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* **145,** 2105–2116.

Schaller, A., Kuhnert, P., de la Puente-Redondo, V.A., Nicolet, J. and Frey, J. (2000). Apx toxins in *Pasteurellaceae* species from animals. *Vet. Microbiol*. **74,** 365–376.

Schindel, C., Zitzer, A., Schulte, B., Gerhards, A., Stanley, P., Hughes, C., Koronakis, V., Bhakdi, S. and Palmer, M. (2001). Interaction of *Escherichia coli* hemolysin with biological membranes. A study using cysteine scanning mutagenesis. *Eur. J. Biochem*. **268,** 800–808.

Schlör, S., Schmidt, A., Maier, E., Benz, R., Goebel, W. and Gentschev, I. (1997). *In vivo* and *in vitro* studies on interactions between the components of the hemolysin (HlyA) secretion machinery of *Escherichia coli*. *Mol. Gen. Genet*. **256,** 306–319.

Schmidt, H., Beutin, L. and Karch, H. (1995). Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun*. **63,** 1055–1061.

Schmidt, H., Maier, E., Karch, H. and Benz, R. (1996). Pore-forming properties of the plasmid-encoded haemolysin of enterohemorrhagic *Escherichia coli* O157:H7. *Eur. J. Biochem*. **241,** 594–601.

Schmitt, L. and Tampe, R. (2002). Structure and mechanism of ABC transporters. *Curr. Opin. Struct. Biol*. **12,** 754–760.

Schmitt, L., Benabdelhak, H., Blight, M.A., Holland, I.B. and Stubbs, M.T. (2003). Crystal structure of the nucleotide-binding domain of the ABC-transporter haemolysin B: identification of a variable region within ABC helical domains. *J. Mol. Biol*. **330,** 333–342.

Schülein, R., Gentschev, I., Mollenkopf, H.-J. and Goebel, W. (1992). A topological model for the haemolysin translocator protein HlyD. *Mol. Gen. Genet*. **234,** 155–163.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Sheahan, K.-L., Cordero, C.L. and Fullner Satchell, K.J. (2004). Identification of a domain within the multifunctional *Vibrio cholerae* RTX toxin that covalently cross-links actin. *Proc. Natl. Acad. Sci. USA* **101,** 9798–9803.

Sheps, J.A., Cheung, I. and Ling, V. (1995). Hemolysin transport in *Escherichia coli*. Point mutants in HlyB compensate for a deletion in the predicted amphiphilic helix region of the HlyA signal. *J. Biol. Chem*. **270,** 14829–14834.

Shewen, P.E. and Wilkie, B.N. (1982). Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infect. Immun*. **35,** 91–94.

Short, E.C. Jr. and Kurtz, H.J. (1971). Properties of the hemolytic activities of *Escherichia coli*. *Infect. Immun*. **3,** 678–687.

Simpson, D.L., Berthold, P. and Taichman, N.S. (1988). Killing of human myelomonocytic leukemia and lymphocytic cell lines by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infect. Immun*. **56,** 1162–1166.

Söderblom, T., Laestadius, A., Oxhamre, C., Aperia, A. and Richter-Dahlfors, A. (2002). Toxin-induced calcium oscillations: a novel strategy to affect gene regulation in target cells. *Int. J. Med. Microbiol*. **291,** 511–515.

Soloaga, A., Ostolaza, H., Goni, F.M. and de la Cruz, F. (1996). Purification of *Escherichia coli* pro-haemolysin, and a comparison with the properties of mature α-haemolysin. *Eur. J. Biochem*. **238,** 418–422.

Soloaga, A., Veiga, M.P., Garcia-Segura, L.M., Ostolaza, H., Brasseur, R. and Goni, F.M. (1999). Insertion of *Escherichia coli* α-haemolysin in lipid bilayers as a non-transmembrane integral protein: prediction and experiment. *Mol. Microbiol*. **31,** 1013–1024.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal α-haemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Stanley, P., Koronakis, V. and Hughes, C. (1991). Mutational analysis supports a role for multiple structural features in the C-terminal secretion signal of *Escherichia coli* haemolysin. *Mol. Microbiol*. **5,** 2391–2403.

Stanley, P.L.D., Diaz, P., Bailey, M.J.A., Gygi, D., Juarez, A. and Hughes, C. (1993). Loss of activity in the secreted form of *Escherichia coli* haemolysin caused by an *rfaP* lesion in core lipopolysaccharide assembly. *Mol. Microbiol*. **10,** 781–787.

Stanley, P., Packman, L.C., Koronakis, V. and Hughes, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science* **266,** 1992–1996.

Stanley, P., Koronakis, V., Hardie, K. and Hughes, C. (1996). Independent interaction of the acyltransferase HlyC with two maturation domains of the *Escherichia coli* toxin HlyA. *Mol. Microbiol*. **20,** 813–822.

Stanley, P., Hyland, C., Koronakis, V. and Hughes, C. (1999). An ordered reaction mechanism for bacterial toxin acylation by the specialized acyltransferase HlyC: formation of a ternary complex with acylACP and protoxin substrates. *Mol. Microbiol*. **34,** 887–901.

Stevens, P.K. and Czuprynski, C.J. (1996). *Pasteurella haemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis *in vitro*. *Infect. Immun*. **64,** 2687–2694.

Strathdee, C.A. and Lo, R.Y.C. (1987). Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli*. *Infect. Immun*. **55,** 3233–3236.

Strathdee, C.A. and Lo, R.Y.C. (1989). Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella haemolytica* leukotoxin determinant. *J. Bacteriol*. **171,** 916–928.

Stroud, R.M., Reiling, K., Wiener, M. and Freymann, D. (1998). Ion-channel-forming colicins. *Curr. Opin. Struct. Biol*. **8,** 525–533.

Sun, Y., Clinkenbeard, K.D., Cudd, L.A., Clarke, C.R. and Clinkenbeard, P.A. (1999). Correlation of *Pasteurella haemolytica* leukotoxin binding with susceptibility to intoxication of lymphoid cells from various species. *Infect. Immun*. **67,** 6264–6269.

Suttorp, N., Flöer, B., Schnittler, H., Seeger, W. and Bhakdi, S. (1990). Effects of *Escherichia coli* hemolysin on endothelial cell function. *Infect. Immun*. **58,** 3796–3801.

Szabo, G., Gray, M.C. and Hewlett, E.L. (1994). Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium- and polarity-dependent manner. *J. Biol. Chem*. **269,** 22496–22499.

Taichman, N.S., Dean, R.T. and Sanderson, C.J. (1980). Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from *Actinobacillus actinomycetemcomitans*. *Infect. Immun*. **28,** 258–268.

Taichman, N.S., Iwase, M., Lally, E.T., Shattil, S.J., Cunningham, M.E. and Korchak, H.M. (1991). Early changes in cytosolic calcium and membrane potential induced by *Actinobacillus actinomycetemcomitans* leukotoxin in susceptible and resistant target cells. *J. Immunol*. **147,** 3587–3594.

Taneike, I., Zhang, H.-M., Wakisaka-Saito, N. and Yamamoto, T. (2002). Enterohemolysin operon of Shiga toxin-producing *Escherichia coli*: a virulence function of inflammatory cytokine production from human monocytes. *FEBS Lett*. **524,** 219–224.

Tascon, R.I., Vazquez-Boland, J.A., Gutierrez-Martin, C.B., Rodriguez-Barbosa, I. and Rodriguez-Ferri, E.F. (1994). The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*: evidence from mutational analysis. *Mol. Microbiol*. **14,** 207–216.

Tatum, F.M., Briggs, R.E., Sreevatsan, S.S., Zehr, E.S., Hsuan, S.L., Whiteley, L.O., Ames, T.R. and Maheswaran, S.K. (1998). Construction of an isogenic leukotoxin deletion mutant of *Pasteurella haemolytica* serotype 1: characterization and virulence. *Microb. Pathog*. **24,** 37–46.

Thanabalu, T., Koronakis, E., Hughes, C. and Koronakis, V. (1998). Substrate-induced assembly of a contiguous channel for protein export from *E. coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO J*. **17,** 6487–6496.

Thompson, S.A. and Sparling, P.F. (1993). The RTX cytotoxin-related FrpA protein of *Neisseria meningitidis* is secreted extracellularly by Meningococci and by HlyBD$^+$ *Escherichia coli*. *Infect. Immun*. **61,** 2906–2911.

Thompson, S.A., Wang, L.L., West, A. and Sparling, P.F. (1993). *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. *J. Bacteriol*. **175,** 811–818.

Thumbikat, P., Briggs, R.E., Kannan, M.S. and Maheswaran, S.K. (2003). Biological effects of two genetically defined leukotoxin mutants of *Mannheimia haemolytica*. *Microb. Pathog*. **34,** 217–226.

Thumbikat, P., Dileepan, T., Kannan, M.S. and Maheswaran, S.K. (2005). Mechanisms underlying *Mannheimia haemolytica* leukotoxin-induced oncosis and apoptosis of bovine alveolar macrophages. *Microb. Pathog*. **38,** 161–172.

Trent, M.S., Worsham, L.M.S. and Ernst-Fonberg, M.L. (1998). The biochemistry of hemolysin toxin activation: characterization of HlyC, an internal protein acyltransferase. *Biochemistry*. **37,** 4644–4652.

Tsai, C.-C., Shenker, B.J., DiRienzo, J.M., Malamud, D. and Taichman, N.S. (1984). Extraction and isolation of a leukotoxin from *Actinobacillus actinomycetemcomitans* with polymyxin B. *Infect. Immun*. **43,** 700–705.

Uhlen, P., Laestadius, A., Jahnukainen, T., Söderblom, T., Backhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A. and Richter-Dahlfors, A. (2000). α-Haemolysin of uropathogenic *E. coli* induces $Ca^{2+}$ oscillations in renal epithelial cells. *Nature* **405,** 694–697.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, Y.-H. and Smith, H.O. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304,** 66–74.

Wagner, W., Vogel, M. and Goebel, W. (1983). Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bacteriol*. **154,** 200–210.

Wandersman, C. and Delepelaire, P. (1990). TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc. Natl. Acad. Sci. USA* **87,** 4776–4780.

Wang, J.F., Kieba, I.R., Korostoff, J., Guo, T.L., Yamaguchi, N., Rozmiarek, H., Billings, P.C., Shenker, B.J. and Lally, E.T. (1998). Molecular and biochemical mechanisms of *Pasteurella haemolytica* leukotoxin-induced cell death. *Microb. Pathog*. **25,** 317–331.

Wang, R., Seror, S.J., Blight, M., Pratt, J.M., Broome-Smith, J.K. and Holland, I.B. (1991). Analysis of the membrane organization of an *Escherichia coli* protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins. *J. Mol. Biol*. **217,** 441–454.

Wang, Z., Clarke, C.R. and Clinkenbeard, K.D. (1999). Role of phospholipase D in *Pasteurella haemolytica* leukotoxin-induced increase in phospholipase $A_2$ activity in bovine neutrophils. *Infect. Immun*. **67,** 3768–3772.

Weingart, C.L., Mobberley-Schuman, P.S., Hewlett, E.L., Gray, M.C. and Weiss, A.A. (2000). Neutralizing antibodies to adenylate cyclase toxin promote phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect. Immun*. **68,** 7152–7155.

Welch, R.A. (1987). Identification of two different hemolysin determinants in uropathogenic *Proteus* isolates. *Infect. Immun*. **55,** 2183–2190.

Welch, R.A. (2001). RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr. Top. Microbiol. Immunol*. **257,** 85–111.

Welch, R.A., Dellinger, E.P., Minshew, B. and Falkow, S. (1981). Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. *Nature* **294,** 665–667.

Wolff, J., Cook, G.H., Goldhammer, A.R. and Berkowitz, S.A. (1980). Calmodulin activates prokaryotic adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **77,** 3841–3844.

Worsham, L.M.S., Trent, M.S., Earls, L., Jolly, C. and Ernst-Fonberg, M.L. (2001). Insights into the catalytic mechanism of HlyC, the internal protein acyltransferase that activates *Escherichia coli* hemolysin toxin. *Biochemistry* **40,** 13607–13616.

Worsham, L.M.S., Langston, K.G. and Ernst-Fonberg, M.L. (2005). Thermodynamics of a protein acylation: activation of *Escherichia coli* hemolysin toxin. *Biochemistry* **44,** 1329–1337.

Yamaguchi, N., Kieba, I.R., Korostoff, J., Howard, P.S., Shenker, B.J. and Lally, E.T. (2001). Maintenance of oxidative phosphorylation protects cells from *Actinobacillus actinomycetemcomitans* leukotoxin-induced apoptosis. *Cell. Microbiol*. **3,** 811–823.

Yamaguchi, N., Kubo, C., Masuhiro, Y., Lally, E.T., Koga, T. and Hanazawa, S. (2004). Tumor necrosis factor alpha enhances *Actinobacillus actinomycetemcomitans* leukotoxin-induced HL-60 cell apoptosis by stimulating lymphocyte function-associated antigen 1 expression. *Infect. Immun*. **72,** 269–276.

Yoo, H.S., Rajagopal, B.S., Maheswaran, S.K. and Ames, T.R. (1995). Purified *Pasteurella haemolytica* leukotoxin induces expression of inflammatory cytokines from bovine alveolar macrophages. *Microb. Pathog*. **18,** 237–252.

Zaitseva, J., Jenewein, S., Jumpertz, T., Holland, I.B. and Schmitt, L. (2005). H662 is the linchpin of ATP hydrolysis in the nucleotide-binding domain of the ABC transporter HlyB. *EMBO J*. **24,** 1901–1910.

Zambon, J.J., DeLuca, C., Slots, J. and Genco, R.J. (1983). Studies of leukotoxin from *Actinobacillus actinomycetemcomitans* using the promyelocytic HL-60 cell line. *Infect. Immun*. **40,** 205–212.

Zaretzky, F.R., Gray, M.C. and Hewlett, E.L. (2002). Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin-filamentous hemagglutinin interaction. *Mol. Microbiol*. **45,** 1589–1598.

Zhang, F., Greig, D.I. and Ling, V. (1993). Functional replacement of the hemolysin A transport signal by a different primary sequence. *Proc. Natl. Acad. Sci. USA* **90,** 4211–4215.

Zhang, F., Yin, Y., Arrowsmith, C.H. and Ling, V. (1995). Secretion and circular dichroism analysis of the C-terminal signal peptides of HlyA and LktA. *Biochem*istry **34,** 4193–4201.

CHAPTER

# 30

# Genetics and phylogeny of RTX cytolysins

*Joachim Frey*

## INTRODUCTION

RTX cytolysins represent a large family of bacterial, pore-forming protein toxins belonging to the type I exoprotein secretion system, which is present in a wide range of Gram-negative bacteria. *In vivo*, RTX toxins induce inflammatory mediators or exert cytotoxic effects, particularly to cells of the host's immune defense, thus provoking necrosis, apoptosis, inflammation, and consequently disease (Czuprynski and Welch, 1995; Welch, 2001). The designation RTX (repeats in the toxin) originates from the common feature, the glycine-rich repeated structures, mostly present in the form of multiple glycine-rich nonapeptides in the structural toxin peptide (Strathdee and Lo, 1989). These repeats have a strong $Ca^{2+}$-binding capacity and are involved in the binding to the host cell membrane. This feature is not only found in cytolysins and hemolysins of pathogenic bacteria, but also in particular in proteases, lipases, bactericides, and nodulation-related proteins, which are all involved in cytotoxicity of pathogenic bacteria or bacteria-host interactions of symbiotic bacteria (Table 30.1). Further common traits of RTX proteins are: (i) the fact that they are generally synthesized as inactive pretoxins and subsequently activated by a specific *cis*-located activator protein together with an acylase of the bacterial host (Hui *et al*., 2000; Issartel *et al*., 1991); and (ii) that they are secreted by particular, *cis*-located type I secretion systems. The close association of RTX cytolysins with type I secretion systems allows the export of the highly membrane-active proteins without damaging the membrane of the bacterial host. RTX cytolysins are therefore encoded on polycistronic operons, including the activator gene, generally termed C, the structural toxin gene termed A, as well as two genes of the type I secretion apparatus encoding the ABC transporter protein B and the export channel protein D. The order of these genes is most often encountered in the arrangement CABD and preceded by a promoter p, which characterizes the RTX operons as genetic entities in a broad spectrum of bacteria (Figure 30.1).

## OPERON STRUCTURES

The hemolysin (Hly) of *Escherichia coli* is the best-analyzed RTX cytolysin, and its operon serves as the archetype of RTX operons (Figure 30.1). The genes of the activator, *hlyC*, the structural hemolysin, *hlyA*, the ABC transporter protein, *hlyB*, and the export channel protein, *hlyD*, are ordered in the *hlyCABD* arrangement that is preceded by the promoter and regulatory genes (Figure 30.1). The *hlyCA* and *hlyBD* genes are separated by a rho-independent transcriptional attenuation sequence, and the *hlyD* gene is followed by a transcriptional stop signal that terminates the *hlyCABD* operon (Figure 30.1). Most hemolysins and leukotoxins, including those of *Actinobacillus actinomycetemcomitans* (*lktA*), *Actinobacillus equuli* (*aqxA*), *Actinobacillus pleuropneumoniae* (*apxIA*, *apxIIA*, *apxIIIA*), *Actinobacillus rossii* (*apxIIA*, *apxIIIA*), *Actinobacillus suis* (*apxIA*, *apxIIA*), Enterohemorrhagic *Escherichia coli* EHEC (*ehxA*), *Mannheimia* (*Pasteurella*) *hemolytica* (*lktA*),

**TABLE 30.1** Structural RTX toxin genes of Gram-negative bacteria

| Gene | Bacterial host | Function | Operon structure* | C+C content | |
|---|---|---|---|---|---|
| | | | | RTX | host |
| *apxI* | *A. pleuropneumoniae* | hemolysin | pCABD | 40 | 42 |
| *apxI* | *A. suis* | hemolysin | pCABD | 40 | 41 |
| *apxII* | *A. pleuropneumoniae* | hemolysin | pCABD | 36 | 42 |
| *apxII* | *A. rossii* | hemolysin | pCABD | 36 | 42 |
| *apxII* | *A. suis* | hemolysin | pCABD | 40 | 41 |
| *apxIII* | *A. pleuropneumoniae* | cytolysin | pCABD | 36 | 42 |
| *apxIII* | *A. pleuropneumoniae* | cytolysin | pCABD | 36 | 42 |
| *apxIV* | *A. pleuropneumoniae* | co-hemolysin, clip-and-link | pORF1A | 40 | 42 |
| *aqxA* | *A. equuli* | hemolysin | pCABD | 39 | 41 |
| *cyaA* | *B. pertussis* | adenylate cyclase-hemolysin | CppABDE | 67 | 68 |
| *cyaA* | *B. bronchiseptica* | adenylate cyclase-hemolysin | CppABDE | 67 | 68 |
| *ehxA* | *E. coli* EHEC | EHEC enterohemolysin | pCABD | 40 | 50 |
| *frpA* | *N. meningitidis* | clip-and-link activity | pORF1A | 44 | 51 |
| *frpC* | *N. meningitidis* | clip-and-link activity | pORF1A | 46 | 51 |
| *hylA* | *E. coli* | hemolysin | pCABD | 41 | 50 |
| *lktA* | *A. actinomycetemcomitans* | leukotoxin | pCABD | 38 | 43 |
| *lktA* | *M. (P.) haemolytica* | leukotoxin | pCABD | 39 | 43 |
| *lktA* | *P. threalosi* | leukotoxin | pCABD | 38 | 42 |
| *lktA* | *M. glucosida* | leukotoxin | pCABD | 39 | 42 |
| *lktA* | *M. varigena* | leukotoxin | pCABD | 38 | 42 |
| *mbxA* | *M. bovis* | leukotoxin | pCABD | 36 | 44 |
| *paxA* | *P. aerogenes* | hemolysin | pCABD | 39 | 42 |
| *paxA* | *P. mairi* | hemolysin | pCABD | 39 | 43 |
| *prtA* | *P. luminescens* | metalloprotease | pIDEFBC | 43 | 45 |
| *prtB* | *E. chrysanthemi* | metalloprotease | pIDEFBC | 55 | 56 |
| *prtc* | *E. chrysanthemi* | metalloprotease | pIDEFBC | 53 | 56 |
| *rtxA* | *V. cholerae* | hemolysin | ACppBD | 49 | 48 |
| *rtxA* | *L. pneumophila* | intracellular survival | | 41 | 39 |

*The operon structure designation refers to Figure 30.1.

*Mannheimia varigena (lktA)*, *Moraxella bovis (mbxA)*, *Pasteurella aerogenes* (*paxA*), and *Pasteurella mairi* (*paxA*) show the *pCABD* operon structure (Figure 30.1, Table 30.1). A minor difference is found in the *A. pleuropneumoniae apxII* operon, which lacks the type I secretion genes B and D. These genes were probably lost during evolution, as indicated by the presence of a residual 300-bp fragment of a potential former *apxIIB* gene downstream from the *apxIIA* gene (Frey *et al*., 1993; Frey, 1995). This loss is, however, compensated by the presence of either a full *apxICABD* operon or a partial *apxIBD* operon in all pathogenic *A. pleuropneumoniae* strains that are able to *trans*-complement the necessary type I secretion functions (Reimer *et al*., 1995; Frey *et al*., 1993). Recently, a complete *apxIICABD* operon has been detected in *Actinobacillus porcitonsillarum*, which further supports the above-mentioned suggestion of a deletion event in *A. pleuropneumoniae* (Kuhnert *et al*., 2005). In addition to the above-mentioned ABC transporter protein B and the export channel protein D, type I secretion systems also require a host outer membrane protein, TolC, encoded by the gene *tolC*, which is located distantly and is genetically not linked to the cytolysin operons pCABD (Andersen *et al*., 2000; Balakrishnan *et al*., 2001; Thanabalu *et al*., 1998). The *tolC* function is also present in strains that are devoid of RTX cytolysins, and TolC proteins seem to be basic outer membrane components in most Gram-negative bacteria. Although *tolC* has not been identified in all bacterial species harboring RTX cytolysins and type I secretion systems, it is expected to be required for all type I secretion systems of the above-named bacteria. While most of the genes of the above-named RTX cytolysins are chromosomally located, it has to be noted that the *E. coli hlyCABD* operon can be both chromosomally and plasmid located (Hacker and Hughes, 1985) and that the EHEC *ehxCABD* operon is placed on the large plasmid of enterohemorrhagic *E. coli* 0157:H7 (Schmidt *et al*., 1996).

Besides the "classical" RTX cytolysins, which closely resemble the *E.coli* hemolysin Hly, several classes of RTX proteins have different biochemical and cytotoxic activities, but all contain glycin-rich repeats

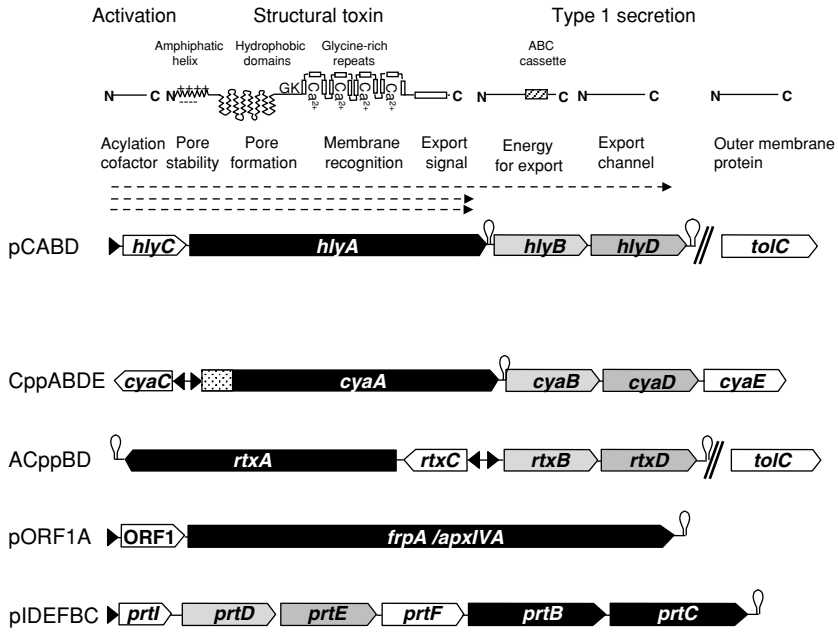



**FIGURE 30.1** Genetic organization and functional domains of RTX operons. The upper part of the figure represents the most common genetic and structural organization of RTX toxins, referred to as pCABD operon, and is mainly based on that of the *E. coli* hemolysin HlyA (Hughes *et al*. 1992; Ludwig *et al*., 1988). The basic functional activities are given on the top line, followed by a schematic statement of the gene products and their major structural characteristics. These domains are annotated. GK indicates the glycin-lysin acylation site. The ATP-binding cassette of the secretion protein B is abbreviated by ABC. N indicates the amino-terminal and C the carboxy-terminal end of the peptides. The four genes of the operon and the unlinked outer membrane gene *tolC* are represented by arrowhead boxes, which indicate the relative length and direction of the coding genes. Black triangles represent promoter sites, and the hairpins show the sites of *rho*-independent transcription termination signals. The dashed arrow above the *hly* gene map represents the direction and length of transcripts. The lower part of the figure shows the genetic organization of the other types of RTX operons, preceded by a short designation, which is reported in Table 30.1. Shading of the different arrowhead boxes was done in analogy to the *hlyC*, *hlyA*, *hlyB*, *hlyD*, and *tolC* genes of the *E. coli* RTX operon. The dotted part of the box of the *cyaA* gene represents the adenylate cyclase moiety.

with $Ca^{2+}$-binding activity. A particular RTX toxin is the calmodulin-sensitive adenylate cyclase-hemolysin CyaA of *Bordetella pertussis*. The *cyaA* gene of the structural toxin shows a significant difference between the guanine-cytosin (G/C) composition of the segment encoding the adenylate cyclase function and the hemolysin function, indicating that this composite protein evolved from a recombination event between two genes, each containing one of the above-named functions. The structure of the *cyaC-cyaABDE* operon shows the particularity that the activator gene C is transcribed on a separate cystron in the opposite direction to the other genes by promoters located between *cyaC* and *cyaA* (Westrop *et al*., 1996; Westrop *et al*., 1997). Moreover, the *TolC* analogue *cyaE* is part of the polycistronic (*cyaABDE*) operon (Figure 30.1).

A further single particular RTX operon is the hemolysin RtxA of *Vibrio cholerae*, which is tightly linked to the cholera toxin prophage (Lin *et al*., 1999; Chattopadhyay and Banerjee, 2003). The genetic organization of this determinant is bicystronic with the *rtxCA* and *rtxBD* type I secretion genes on two separated transcriptional units that are read in opposite directions from two adjacent promoters (Figure 30.1). While the genetic organization of the *V. cholerae* hemolysin determinant is characteristic for RTX cytolysins, this hemolysin lacks the canonical glycin-rich nonapeptide repeats and possesses instead an 18-residue glycine/aspartate-rich repeat that seems to be involved in $Ca^{2+}$-binding, similar to the alkaline protease ArpA of *Pseudomonas aeruginosa* (Baumann *et al*., 1993; Duong *et al*., 1992).

Several bacterial virulence attributes exhibit structural and functional features of RTX toxins, in particular, the glycine-rich nonapeptide repeats or glycine/aspartate-rich repeats with $Ca^{2+}$-binding capacity, yet with biochemical activities and genetic structures that are different from the hemolysins/cytolysins described above. Among them are the large, 200–300-kDa RTX proteins ApxIV of *A. pleuropneumoniae*, as well as FrpC and FrpA of *Neisseria meningitidis*. These proteins show both a novel calcium-dependent processing and cross-linking (clip and link) activity (Osicka *et al*., 2004), and they are known to induce high levels of

serum antibodies during infection (Thompson *et al*., 1993; Osicka *et al*., 2004; Schaller *et al*., 1999; Dreyfus *et al*., 2004). Recombinant ApxIV shows a co-hemolytic activity to porcine and ovine erythrocytes in synergy with sphyingomyelinase, similar to certain RTX cytolysins described above (Schaller *et al*., 1999). The genetic setup of this class of clip and link activity RTX proteins, however, strongly differs from the other RTX toxins and is characterized by a bicistronic operon, including a short, open-reading frame (ORF1) of about 450 base pairs (bp) followed by the structural RTX toxin gene A of 6000–7000 bp (Figure 30.1). The 450 bp ORF1 upstream *frpA*, *frpC*, and *apxIV* shows no similarity to the activator gene *hlyC* or other typical C-activators of RTX cytolysins, and the function of this potential co-factor is unknown. However, it has been shown that its presence is required for the co-hemolytic activity of ApxIVA (Schaller *et al*., 1999). The large RTX proteins FrpC, FrpA, and ApxIV carry a C-terminal 60 amino-acid structure, revealing several features that are conserved among secreted RTX proteins (Thompson and Sparling, 1993). FrpA can indeed be secreted by *E. coli* harboring *hlyBD* genes (Thompson and Sparling, 1993). However, no type I secretion genes have been evidenced in the vicinity of *frpA*, *frpC*, or *apxIV*, indicating that the corresponding type I secretion genes are unlinked in *N. meningitidis* and *A. pleuropneumoniae*.

A particular class of RTX proteins is constituted by a group of metalloproteases, including the PrtB and PrtC zinc metalloproteases of *Erwinia chrysanthemi* (Delepelaire and Wandersman, 1989), the zinc metalloprotease PrtA of *Photorhabdus luminescens* (Bowen *et al*., 2003), as well as the alkaline protease AprA of *Pseudomonas aeruginosa* (Duong *et al*., 1992) and the metalloprotease SrpA of *Serratia marcescens* (Suh and Benedik, 1992; Letoffe *et al*., 1991). The genetic structure of this class of RTX proteins is given in Figure 30.1. In *E. chrysanthemi*, this operon is composed by gene *prtI* encoding an inhibitor protein, which protects the bacterial host from detrimental effects of the protease, the three genes *prtD*, *prtE*, and *prtF* encoding transporter genes for secretion, and the two metalloprotease genes *prtB* and *prtC* (Figure 30.1). It has to be noted that the gene designations of these RTX protease operons are less consistent than those of the "classical" RTX cytolysins. Hence, in *P. luminescens*, the operon metalloprotease is named *prtA*, followed by the inhibitor gene *prtI* and then by type I secretion genes *prtB*, *prtC*, and *prtD*. Different gene designations are also used for three metalloprotease genes of *E. chrysanthemi* EC16, which are named *prtA*, *prtB*, and *prtC* (Dahler *et al*., 1990) and which all encode metalloproteases and not secretory functions.

## REGULATION OF GENE EXPRESSION OF RTX CYTOLYSINS

The regulation of expression of the *hlyCABD* operon has been investigated in detail (Bailey *et al*., 1997; Koronakis *et al*., 1989). It is important to consider that the *hlyCABD* operon contains two different groups of genes whose gene products are needed at completely different quantities. On the one hand, the *hlyA* product, which is secreted as HlyA hemolysin-cytolysin, and its activator, the *hlyC* gene product, are needed in high amounts at particular steps during the infectious process. On the other hand, the gene products of the type I secretion genes *hlyB* and *hlyD* are integral membrane components that, once inserted in the bacterial membranes, fulfill their function, and hence their gene expression does not need to be up-regulated at particular moments of the infection. Furthermore, the number of type I secretion pores or channels is expected to be limited in a bacterium in order to ensure its membrane integrity. In the *hlyCABD* operon, the structural toxin- and activator-genes *hlyA* and *hlyC* are contiguous and co-transcribed with the export genes *hlyB* and *hlyD* from promoters proximal to *hlyC*, but toxin synthesis and the export functions can be uncoupled by termination of the great majority of *hlyC* and *hlyA* transcripts within the intergenic poly U-sequence of a rho-independent terminator located between hlyA and the translation start of *hlyB* (Koronakis *et al*., 1989; Koronakis and Hughes, 1988; Welch and Pellett, 1988). Transcription of the *hlyCABD* operon requires the *rfaH* (*hlyT*) locus, a specific regulator of transcript elongation, and a 5′ *cis*-acting *ops* element, which is also involved in the regulation of transcription of genes of F-pilus and lipolysaccharide biosynthesis genes of *E. coli*, *Shigella flexneri*, *Yersinia enterocolitica*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Bailey *et al*., 1997; Bailey *et al*., 1992; Bailey *et al*., 1996). Uncoupling of transcription of the RTX toxin genes CA from the type I secretion genes BD by transcription attenuation by a rho-independent transcription termination locus has also been evidenced for the *apxICABD* operon in *A. pleuropneumoniae* (Gygi *et al*., 1992). Operon apxICABD, however, does not contain the 5′, *cis*-acting *ops* element. Expression of the *apxICABD* operon is strongly enhanced by extracellular free $Ca^{2+}$ ions at activities above 3mM, which increases ApxI production of *A. pleuropneumoniae* serotype 1 strains by a factor of 300 (Frey and Nicolet, 1988). The promoter of the *apxICABD* has been mapped by primer extension experiments. It revealed a particular ACAAAT (–44) box upstream the –35 sequence and an unusually long A+U rich leader sequence of the cor-

responding mRNA (Frey *et al*., 1994). While the promoter sequences of most of the RTX operons with pCABD structure seem to differ strongly, these operons all share the rho-independent transcriptional stop signal between the A and B genes, which seems to be crucial for an optimal repartition between the *CA* and the *CABD* transcripts.

Expression of the *frpA* and *frpC* genes in *N. meningitides* was shown to be iron-regulated, hence the designation *frp* (Fe-regulated protein) (Thompson *et al*., 1994). When *N. meningitidis* is grown under iron-depleted conditions in growth medium, the *frpA* and *frpC* genes are expressed, while they are repressed in the presence of $Fe^{2+}$-ions in the growth medium. This regulation is expected to depend on the ferric uptake regulation mechanism "Fur" since recognition sequence for regulatory Fur-proteins, the Fur-box, has been identified in the promoter sequences upstream ORF1, which is located upstream *frpA* and *frpC* and is co-transcribed with these genes (Thompson *et al*., 1993). Interestingly, the *apxIV* operon, which shows the same genetic structure as the *frpA* and *frpC* operons (Figure 30.1), is not regulated by $Fe^{2+}$ (Schaller *et al*., 1999). ApxIVA is not produced when *A. pleuropneumoniae* is grown in a growth medium under several different conditions and not in the absence or in the presence of many co-factors tested, including $Fe^{2+}$. However, since pigs infected with *A. pleuropneumoniae* show a rapid sero-conversion for ApxIVA, it is concluded that the *apxIVA* gene is specifically induced *in vivo* during the infection by a mechanism currently still unknown (Schaller *et al*., 1999).

## PHYLOGENY AND THE SPREAD OF RTX CYTOLYSIN GENES

The genetic relationship of the different RTX toxins was calculated from the similarity of analysis of the nucleotide sequences of the structural toxin A using the Jukes-Canter algorithm (Jukes and Cantor, 1969) and cluster analysis. A phylogenetic unrooted tree was built by the neighbor-joining method as described by (Chiesa *et al*., 1993) (Figure 30.2). Based on their genetic relationship, RTX toxins can be grouped in three main clusters (Figure 30.2). The said clusters reflect some of the main *in vitro* properties of these toxins, as well as their basic operon structures. Thus, cluster 1 exclusively contains hemolysins/cytolysins that have the typical *pCABD* operon structure mostly found in *Pasteurellaceae* but also in *E. coli* and *M. bovis*. All these A-genes also show a characteristically low GC-content of their nucleotide composition as a further common feature, which is close to that of the species of the family *Pasteurellaceae* (Table 30.1). While cluster 1 can be further subdivided phylogenetically into two subclusters, this further subdivision cannot be attributed to a particular function or gene structure. Both subclusters each represent a relatively homogenous group of structural RTX toxin genes with genetic distances below 50% (Figure 30.2). In contrast, some of the host bacteria harboring these RTX genes are phylogenetically distant from each other. In particular, *E. coli*, which harbors *hlyA*, referred to as the archetype of RTX toxin genes, is phenotypically only distantly related to the species *A. pleuropneumoniae*, a member of the *Pasteurellaceae* harboring *apxA*. However, *hlyA* is phylogenetically most closely related to *apxI* (Figure 30.2) and shows very similar phenotypic characteristics under *in vitro* conditions, too. Moreover, the GC content of *hlyA,* which is 41%, strongly differs from that of the *E. coli* genome (50%), but is close to that of most *Pasteurellaceae* genomes (41%–43%) (Table 30.1). It clearly indicates that *hlyA*, and with it the entire *hlyCABD* operon, was horizontally transferred, most probably from a species of the *Pasteurellaceae* family. This also seems to be true for the *mbxA* gene of *M. bovis*, a member of the family of *Moraxellaceae* (order *Pseudomonadales*). The *mbxA* gene is most closely related to the *aqxA* hemolysin of *A. equuli*, an equine pathogen belonging to the family of *Pasteurellaceae*.

The second cluster of RTX genes contains exclusively RTX protease genes, including the metalloprotease genes *prtB* and *prtC* of *E. chrysanthemi* and *prtA* of *P. luminescens*, as well as the clip and link protease genes *frpC* and *frpA* of *N. meningitidis* and *apxIV* of *A. pleuropneumoniae* (Figure 30.2). While the genetic organization of the metalloproteases includes secretory genes, the operons and the clip and link proteases are devoid of export functions. On the whole, all these protease genes are phylogenetically distant from the hemolysin-cytolysin genes found in cluster 1, which reflects not only the different biological activities of their gene products, but also a generally higher GC content. This group of RTX genes generally also has a GC content corresponding to the genomic GC content of their respective hosts (Table 30.1). Both the GC contents and the phylogenetic relationship among members of protease-RTX genes of this cluster allow speculation that their respective hosts adopted these genes earlier in evolution than the "classical" RTX operons.

The third cluster of RTX genes (Figure 30.2) contains rather heterogeneous members. Although, to some extent, their operon organizations seem to be composed of blocks of the "classic" pCABD operons, the structural toxin genes are distantly related to the latter and are composed of GC contents that are significantly

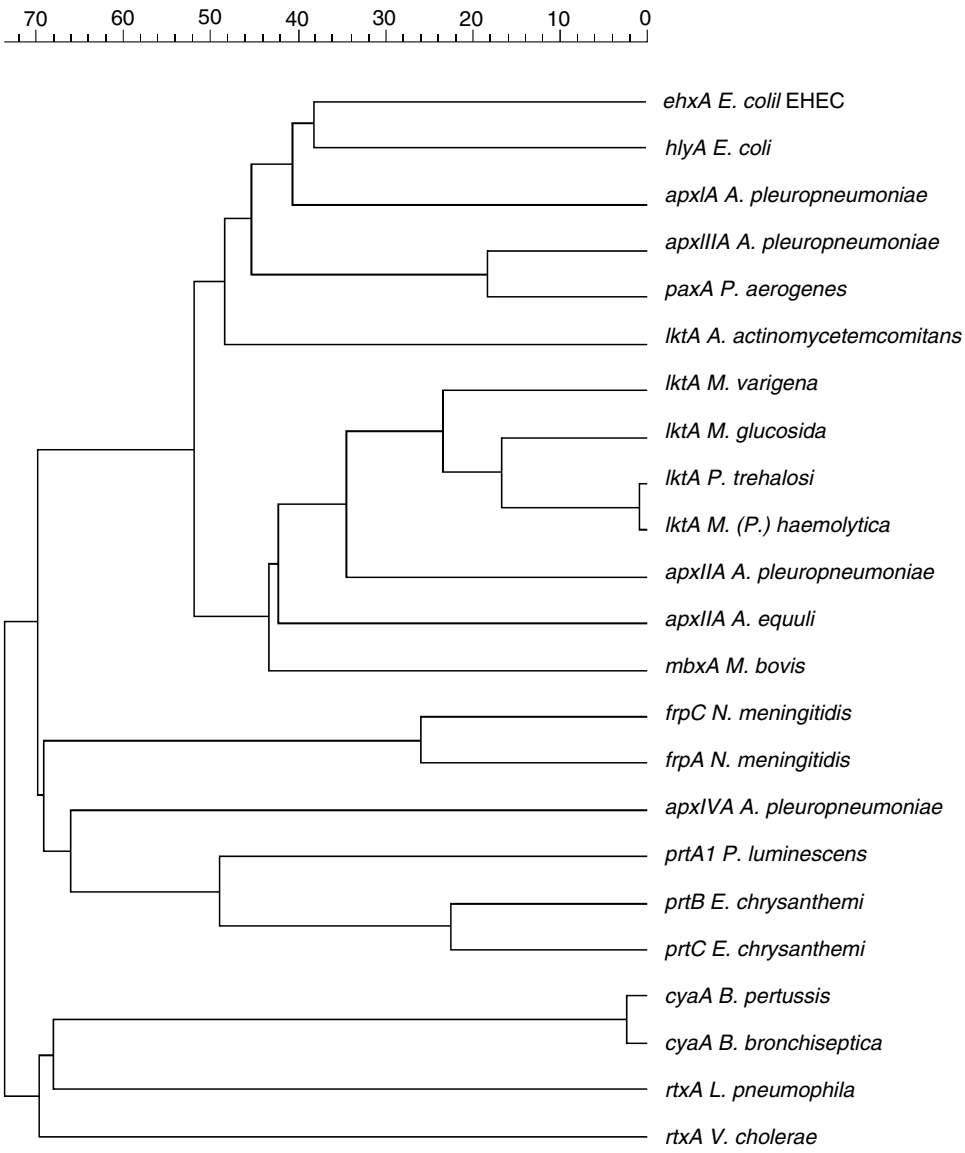



**FIGURE 30.2** Phylogenetic relationship of structural RTX toxin genes of various Gram-negative bacteria. The scale on the top represents the genetic distances in percent nucleotide difference. For the calculation of the distance matrix, only the hemolysin moiety of the *cyaA* genes has been used.

higher and more closely adapted to the genomes of their hosts, which represent members of distant bacterial families (Table 30.1).

## CONCLUSION

Genes encoding RTX toxins are found among a broad diversity of Gram-negative bacterial species. They are most commonly found in species of the *Pasteurellaceae* family from where the typical RTX genes with the pCABD operon organization (Figure 30.1) seem to have developed. This type of RTX genes, which encode for hemolysins and cytolysins, seem to have spread horizontally into other bacterial species such as *Escherichia coli*, where they are found in uropathogenic and enterohemorrhagic *E. coli*, and into *Moraxella bovis*. The two phylogenetically distantly related groups of RTX toxins do not only show different phenotypic and biochemical functions, but are also organized in different operon structures. While they all contain glycin-rich repeated arrays with $Ca^{2+}$-binding capacity in their structural toxin peptides, which group them to the family of RTX toxins, their different genetic, phylogenetic, and functional features indicate that they should be treated as a different group or subgroup of toxins.

## REFERENCES


Andersen, C., Hughes, C. and Koronakis, V. (2000). Channel vision. Export and efflux through bacterial channel-tunnels. *EMBO Rep*. **1,** 313–318.

Bailey, M.J., Hughes, C. and Koronakis, V. (1997). RfaH and the *ops* element, components of a novel system controlling bacterial transcription elongation. *Mol. Microbiol*. **26,** 845–851.

Bailey, M.J., Koronakis, V., Schmoll, T. and Hughes, C. (1992). *Escherichia coli* HlyT protein, a transcriptional activator of hemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. *Mol. Microbiol*. **6,** 1003–1012.

Bailey, M.J.A., Hughes, C. and Koronakis, V. (1996). Increased distal gene transcription by the elongation factor RfaH, a specialized homologue of NusG. *Mol. Microbiol*. **22,** 729–737.

Balakrishnan, L., Hughes, C. and Koronakis, V. (2001). Substrate-triggered recruitment of the TolC channel-tunnel during type I export of hemolysin by *Escherichia coli*. *J. Mol. Biol*. **313,** 501–510.

Baumann, U., Wu, S., Flaherty, K.M. and McKay, D.B. (1993). Three-dimensional structure of the alkaline protease of *Pseudomonas aeruginosa*: a two-domain protein with a calcium-binding parallel beta roll motif. *EMBO J*. **12,** 3357–3364.

Bowen, D.J., Rocheleau, T.A., Grutzmacher, C.K., Meslet, L., Valens, M., Marble, D., Dowling, A., Ffrench-Constant, R. and Blight, M.A. (2003). Genetic and biochemical characterization of PrtA, an RTX-like metalloprotease from *Photorhabdus*. *Microbiology* **149,** 1581–1591.

Chattopadhyay, K. and Banerjee, K.K. (2003). Unfolding of *Vibrio cholerae* hemolysin induces oligomerization of the toxin monomer. *J. Biol. Chem*. **278,** 38470–38475.

Chiesa, C., Pacifico, L. and Ravagnan, G. (1993). Identification of pathogenic serotypes of *Yersinia enterocolitica*. *J. Clin. Microbiol*. **31,** 2248.

Czuprynski, C.J. and Welch, R.A. (1995). Biological effects of RTX toxins: The possible role of lipopolysaccharide. *Trends Microbiol*. **3,** 480–483.

Dahler, G.S., Barras, F. and Keen, N.T. (1990). Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. *J. Bacteriol*. **172,** 5803–5815.

Delepelaire, P. and Wandersman, C. (1989). Protease secretion by *Erwinia chrysanthemi*: Protease B and C are synthesized and secreted as zymogens without a signal peptide. *J. Biol. Chem*. **264,** 9083–9089.

Dreyfus, A., Schaller, A., Nivollet, S., Segers, R.P., Kobisch, M., Mieli, L., Soerensen, V., Hussy, D., Miserez, R., Zimmermann, W., Inderbitzin, F. and Frey, J. (2004). Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development, and prevalidation of the ApxIV ELISA. *Vet. Microbiol*. **99,** 227–238.

Duong, F., Lazdunski, A., Cami, B. and Murgier, M. (1992). Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. *Gene* **121,** 47–54.

Frey, J. (1995). Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. *Trends Microbiol*. **3,** 257–261.

Frey, J., Bosse, J.T., Chang, Y.F., Cullen, J.M., Fenwick, B., Gerlach, G.F., Gygi, D., Haesebrouck, F., Inzana, T.J., Jansen, R., Kamp, E.M., Macdonald, J., MacInnes, J.I., Mittal, K.R., Nicolet, J., Rycroft, A.N., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Struck, D.K., Vandenbosch, J.F., Willson, P.J. and Young, R. (1993). *Actinobacillus pleuropneumoniae* RTX-toxins—Uniform designation of hemolysins, cytolysins, pleurotoxin, and their genes. *J. Gen. Microbiol*. **139,** 1723–1728.

Frey, J., Haldimann, A., Nicolet, J., Boffini, A. and Prentki, P. (1994). Sequence analysis and transcription of the *apxI* operon (hemolysin I) from *Actinobacillus pleuropneumoniae*. *Gene* **142,** 97–102.

Frey, J. and Nicolet, J. (1988). Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by $Ca^{2+}$. *Infect. Immun*. **56,** 2570–2575.

Gygi, D., Nicolet, J., Hughes, C. and Frey, J. (1992). Functional analysis of the $Ca^{2+}$-regulated hemolysin I operon of *Actinobacillus pleuropneumoniae* serotype 1. *Infect. Immun*. **60,** 3059–3064.

Hacker, J. and Hughes, C. (1985). Genetics of *Escherichia coli* hemolysin. *Curr. Top. Microbiol. Immunol*. **118,** 139–162.

Hughes, C., Stanley, P. and Koronakis, V. (1992). *E. coli* hemolysin interactions with prokaryotic and eukaryotic cell membranes. *Bioessays* **14,** 519–525.

Hui, D., Morden, C., Zhang, F. and Ling, V. (2000). Combinatorial analysis of the structural requirements of the *Escherichia coli* hemolysin signal sequence. *J. Biol. Chem*. **275,** 2713–2720.

Issartel, J.P., Koronakis, V. and Hughes, C. (1991). Activation of *Escherichia coli* prohemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* **351,** 759–761.

Jukes, T.H. and Cantor, C.R. (1969). Evolution of protein molecules. In: *Mammalian Protein Metabolism* (ed. H.N. Munro), Vol. 3. pp. 21–132. Academic Press, New York.

Koronakis, V., Cross, M. and Hughes, C. (1989). Transcription antitermination in an *Escherichia coli* hemolysin operon is directed progressively by cis-acting DNA sequences upstream of the promoter region. *Mol. Microbiol*. **3,** 1397–1404.

Koronakis, V. and Hughes, C. (1988). Identification of the promoters directing *in vivo* expression of hemolysin genes in *Proteus vulgaris* and *Escherichia coli*. *Mol. Gen. Genet*. **213,** 99–104.

Kuhnert, P., Schlatter, Y. and Frey, J. (2005). Characterization of the type I secretion system of the RTX toxin ApxII in *Actinobacillus porcitousillarum*.*Vet. Microbiol*. **107,** 225–232.

Letoffe, S., Delepelaire, P. and Wandersman, C. (1991). Cloning and expression in *Escherichia coli* of the Serratia marcescens metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. *J. Bacteriol*. **173,** 2160–2166.

Lin, W., Fullner, K.J., Clayton, R., Sexton, J.A., Rogers, M.B., Calia, K.E., Calderwood, S.B., Fraser, C. and Mekalanos, J.J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proc. Natl. Acad. Sci. USA* **96,** 1071–1076.

Ludwig, A., Jarchau, T., Benz, R. and Goebel, W. (1988). The repeat domain of *Escherichia coli* hemolysin (HlyA) is responsible for its $Ca^{2+}$-dependent binding to erythrocytes. *Mol. Gen. Genet*. **214,** 553–561.

Osicka, R., Prochazkova, K., Sulc, M., Linhartova, I., Havlicek, V. and Sebo, P. (2004). A novel "clip-and-link" activity of repeat in toxin (RTX) proteins from Gram-negative pathogens. Covalent protein cross-linking by an Asp-Lys isopeptide bond upon calcium-dependent processing at an Asp-Pro bond. *J. Biol. Chem*. **279,** 24944–24956.

Reimer, D., Frey, J., Jansen, R., Veit, H.P. and Inzana, T.J. (1995). Molecular investigation of the role of ApxI and ApxII in the virulence of *Actinobacillus pleuropneumoniae* serotype 5. *Microb. Pathog*. **18,** 197–209.

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., MacInnes, J.I., Segers, R.P.A.M. and Frey, J. (1999). Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* **145,** 2105–2116.

Schmidt, H., Kernbach, C. and Karch, H. (1996). Analysis of the EHEC hly operon and its location in the physical map of the large plasmid of enterohemorrhagic *Escherichia coli* O157:H7. *Microbiology* **142,** 907–914.

Strathdee, C.A. and Lo, R.Y. (1989). Cloning, nucleotide sequence, and characterization of genes encoding the secretion function of the *Pasteurella hemolytica* leukotoxin determinant. *J. Bacteriol*. **171,** 916–928.

Suh, Y. and Benedik, M.J. (1992). Production of active *Serratia marcescens* metalloprotease from *Escherichia coli* by alpha-hemolysin HlyB and HlyD. *J. Bacteriol*. **174,** 2361–2366.

Thanabalu, T., Koronakis, E., Hughes, C. and Koronakis, V. (1998). Substrate-induced assembly of a contiguous channel for protein export from *E.coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO J*. **17,** 6487–6496.

Thompson, S.A. and Sparling, P.F. (1993). The RTX cytotoxin-related FrpA protein of *Neisseria meningitidis* is secreted extracellularly by meningococci and by HlyBD$^+$ *Escherichia coli*. *Infect. Immun*. **61,** 2906–2911.

Thompson, S.A., Wang, L.L. and Sparling, P.F. (1993). Cloning and nucleotide sequence of *frpC*, a 2nd gene from *Neisseria meningitidis* encoding a protein similar to RTX cytotoxins. *Mol. Microbiol*. **9,** 85–96.

Thompson, S.A., Wang, L.L., West, A. and Sparling, P.F. (1994). *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. *J. Bacteriol*. **175,** 811–818.

Welch, R.A. (2001). RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Curr. Top. Microbiol. Immunol*. **257,** 85–111.

Welch, R.A. and Pellett, S. (1988). Transcriptional organization of the *Escherichia coli* hemolysin genes. *J. Bacteriol*. **170,** 1622–1630.

Westrop, G., Hormozi, K., daCosta, N., Parton, R. and Coote, J. (1997). Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *J. Bacteriol*. **179,** 871–879.

Westrop, G.D., Hormozi, E.K., Da Costa, N.A., Parton, R. and Coote, J.G. (1996). *Bordetella pertussis* adenylate cyclase toxin: proCyaA and CyaC proteins synthesized separately in *Escherichia coli* produce active toxin *in vitro*. *Gene* **180,** 91–99.

CHAPTER

# 31

# The family of two-component cytolysins of *Serratia* and other bacteria

*Volkmar Braun and Ralf Hertle*

## INTRODUCTION

This report is a follow-up of the review in the former *Comprehensive Sourcebook of Bacterial Protein Toxins* (Braun and Hertle, 1999). The previously described major findings will be briefly summarized, and new findings will be discussed in more detail. For a complete list of older references, the reader is referred to the literature cited in the earlier review.

The *Serratia marcescens* hemolysin (cytolysin; ShlA) has several novel features: secretion across the outer membrane by a specific protein that also converts the polypeptide into an active hemolysin and the dependence of the activity on phospholipids, specifically on phosphatidylethanolamine and phosphatidylserine. The ShlA secretion mechanism has become the paradigm of over 100 predicted exoproteins, few of which have been genetically and biochemically characterized. The *S. marcescens* cytolysin is the first example of a protein exotoxin determined by two genes; *shlA* encodes the cytolysin, and *shlB* encodes an outer membrane protein required for ShlA secretion and activation (Braun *et al*., 1987; Poole *et al*., 1988). These two adjacent genes are transcribed in the order *shlB shlA* from an iron-regulated promoter upstream of *shlB*. In *Escherichia coli* transformed with the *shlBA* genes, iron regulation occurs via the Fur protein, which when loaded with $Fe^{2+}$, acts as a transcriptional repressor (Poole and Braun, 1988).

Over 90% of tested clinical isolates of *S. marcescens* and *S. liquefaciens* synthesize the hemolysin. Cytolysins with amino acid sequences similar to ShlA/B have been described in *Proteus mirabilis*, *Haemophilus ducreyi*, *Edwardsiella tarda*, and *Photorhabdus luminescens* (Brillard *et al*., 2002), and encoding genes are found in the genome sequences of *Yersinia pestis*, *Chromobacterium violaceum*, and *Xylella fastidiosa* (Figure 31.1 A, B)

Mature ShlA is composed of 1,578 residues, which can be truncated to an N-terminal fragment of 238 residues that is still secreted, but is non-hemolytic. Progressive deletion of the C-terminus of ShlA gradually reduces hemolytic activity; the smallest active fragment of 104 kDa exhibits only 3% of wild-type activity and still integrates into erythrocyte membranes. An N-terminal nonhemolytic 72-kDa fragment binds to but does not insert into the erythrocyte membrane. ShlA remains active in 6 M urea and is cleaved by trypsin (0.3 M urea) into a major 89-kDa fragment. Upon integration into the erythrocyte membrane, ShlA becomes trypsin-resistant from outside the erythrocyte membrane, but is cleaved into 143- and 14.5-kDa fragments when inside-out erythrocyte vesicles are treated with trypsin. Upon longer exposure, the 143-kDa fragment is further cleaved into 138-, 58-, and 38-kDa fragments. In the presence of Triton X-100, which could partially reflect the hydrophobic environment in erythrocyte membranes, trypsin cleaves ShlA into fragments of 138-, 89-, and 58-kDa. The data suggest that ShlA is an integral membrane protein with no trypsin-sensitive sites exposed at the erythrocyte surface, but with trypsin cleavage sites exposed at the cytosolic side. In the membrane, ShlA assumes a conformation that differs from that in buffer.

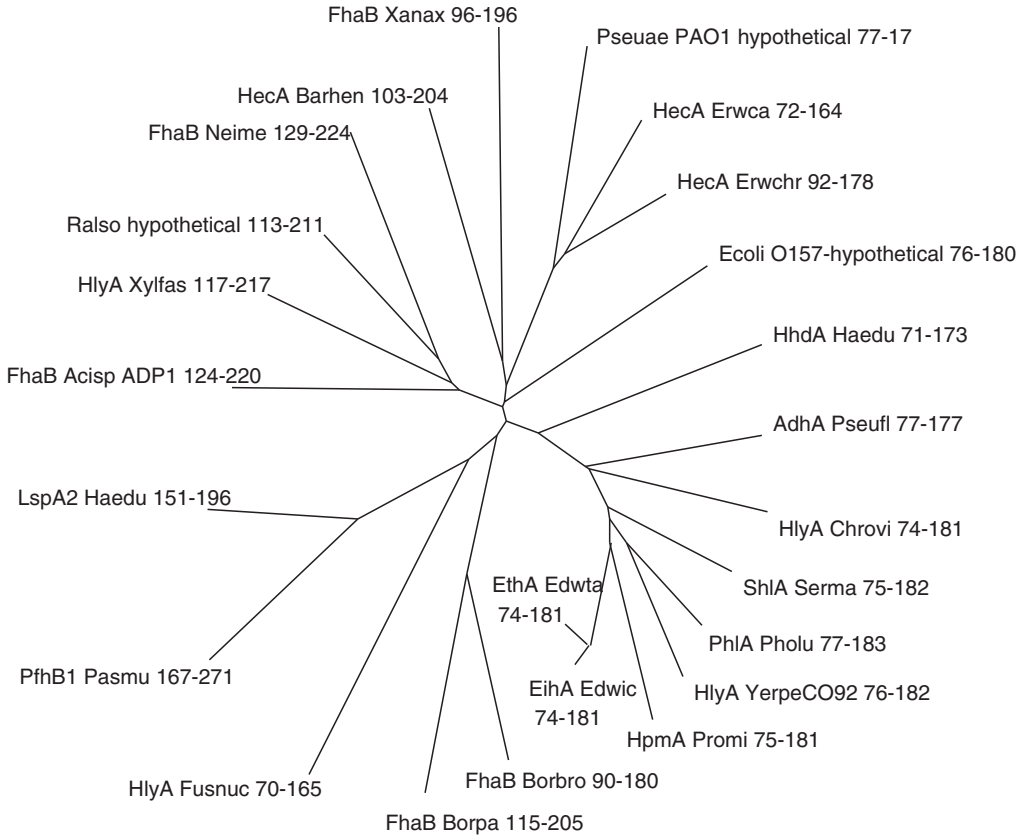



**FIGURE 31.1** A dendrogram of proteins related to the *S. marcescens* hemolysin ShlA. Sequences of the activation and secretion domain were aligned (108 amino acids for ShlA, as shown in Figure 31.2). The first and last amino acid residues of the homologous sequences are indicated after the strain name. *Serratia marcescens* (ShlA Serma) and the related hemolysins of *Edwardsiella tarda* PAO1 (Pseuae PAO1 hypothetical), *Pseudomonas fluorescens* (AdhB Pseufl), *Acinetobacter* sp. ADP1 (FhaC Acisp ADP1l), *Xanthomonas axonopodis* (FhaC Xanax), the large secretory protein from *Haemophilus ducreyi* and *Pasteurella multocida* (LspB Haedu and LspB Pasmu, respectively), and *Escherichia coli* O157:H7 (Ecoli O157-hypothetical) as determined by the CLUSTAL-W program, are shown.

ShlA forms pores of a limited size range in the erythrocyte membrane that cause osmolysis. Added maltoheptaose (molecular weight 1,152) largely prevents osmolysis because it does not diffuse through the ShlA pores and thus counterbalances the internal osmotic pressure of the erythrocytes.

## PHOSPHOLIPIDS ARE REQURED FOR ShlA ACTIVITY

No active ShlA is formed in the absence of ShlB. Inactive ShlA is not secreted into the culture medium, but rather stays in the periplasm. A lysate of cells that synthesize inactive ShlA (ShlA$^*$) and secrete it into the periplasm via the Sec system mixed with a lysate of cells having ShlB in the outer membrane results in hemolytic ShlA. However, highly purified ShlA$^*$ mixed with ShlB fails to yield hemolytic ShlA. The missing component in the mixture is phosphatidylethanolamine, the major phospholipid in the *S. marcescens* outer membrane, which when added to the latter mixture results in active ShlA. Incubation with phospholipase A2, which cleaves the fatty acid at the C2 position of the glycerol residue and which is devoid of protease activity, inactivates active ShlA and prevents activation of ShlA$^*$ by ShlB. These results show that ShlB activates ShlA$^*$, secretion across the outer membrane is not required for activation, and ShlA activity requires phospholipids.

Previously, we have reported that phospholipid is not covalently bound, but is rather tightly bound to ShlA and remains associated with ShlA during SDS-PAGE. Electrophoresis experiments with a higher resolution in the 160 kDa region revealed that the former association of radioactivity with ShlA is a contaminant of the radiolabeled phospholipid preparations (G. Walker, thesis). To determine the region in ShlA to which phospholipid is bound, complementation and binding assays have been used. A deletion derivative

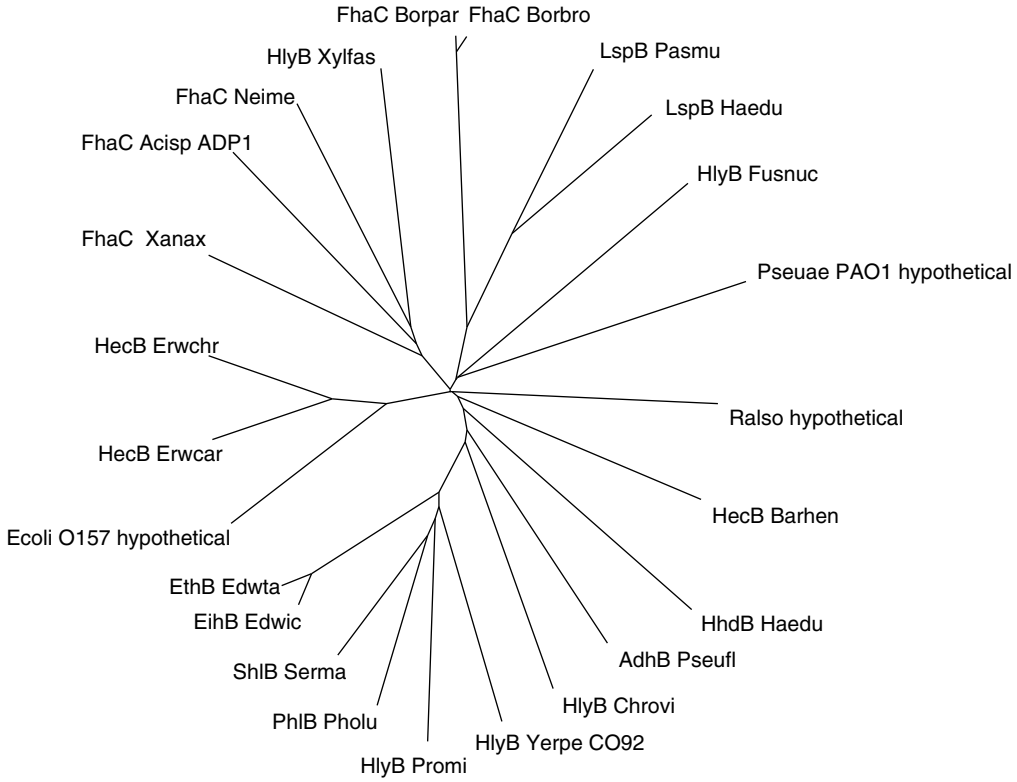



**FIGURE 31.1, Cont'd.**

lacking residues 3–97 of mature ShlA, ShlAΔ3–97, is inactive and not secreted by ShlB into the culture medium. It can be complemented to hemolytic ShlA by N-terminal ShlA fragments, the shortest of which consists of residues 1–238 (ShlA238). This N-terminal fragment is secreted by ShlB and therefore contains the information for specific recognition and secretion by ShlB. It also contains the activation domain. The N-terminal fragments ShlA238, ShlA269, and ShlA667 do not complement phospholipase-A2-treated ShlAΔ3–97, but ShlA1003 and ShlA1144 complement. Treatment of ShlA1003 and ShlA1144 with phospholipase A2 reduces the complementing activity. Phospholipids are associated with ShlA1003 and ShlA1144 and restore the activity of phospholipase-treated ShlAΔ3–97 (G. Walker thesis, 2001). However, radioactive phosphatidylethanolamine binds to ShlA268 (Hertle, unpublished observation), which suggests that phospholipids bind to the N-terminal and the central part of ShlA, or that the ShlA fragment must have a certain length to fold, such that phospholipid binding sites are formed. It has been estimated that three to four phospholipid molecules bind to one ShlA molecule over its entire length. This number of phospholipid molecules is not sufficient to form a micelle around ShlA. Rather, it indicates specific phospholipid binding sites in ShlA.

## ACTIVATION OF ShlA BY A CONFORMATIONAL CHANGE

Hemolytic ShlA incubated with SDS at 20°C forms two bands after SDS-PAGE and one band if first incubated at 95°C. The structural change revealed by the different electrophoretic mobilities is not observed with inactive ShlA, which forms one band at both temperatures. Structural differences between active and inactive ShlA are also revealed by strong differences in trypsin sensitivities. Hemolytic ShlA exhibits a single trypsin cleavage site after incubation at 0°C, yielding two fragments of 60 and 100 kDa, whereas inactive ShlA is completely degraded by trypsin. Electrospray mass spectrometry has determined the same molecular weight for the two proteins, which corresponds exactly with the calculated value. Activation does not change the chemical structure of ShlA, but rather the conformation.

ShlA aggregates and rapidly precipitates, which can be prevented by 6 M urea in which ShlA is physically

stable and active. An N-terminal fragment of 242 residues, ShlA242, is soluble in buffer lacking urea. ShlA242 complements non-hemolytic ShlA and ShlAΔ3–97 to active ShlA when secreted by ShlB and displays no complementation when it is synthesized by cells lacking ShlB. To determine ShlA activation, ShlA242 has been used because it is easier to study experimentally. Four ShlA242 samples have been studied: ShlA242 remaining in the cytoplasm owing to the lack of a signal sequence for secretion; ShlA242 in the periplasm synthesized in the absence of ShlB; ShlA242 in the culture supernatant exported through ShlB; and ShlA242 exported, but not activated by an ShlB mutant that contains a tetrapeptide insertion after residue 136, ShlB136ARSG (Yang *et al*., 2000). Electrospray mass spectroscopy shows that the four derivatives have the same molecular weight, which corresponds to the calculated molecular weight. Upon native polyacrylamide gel electrophoresis (no heating, SDS, mercaptoethanol), the three inactive ShlA242 derivatives display a much slower electrophoretic mobility than active ShlA242. Circular dichroism reveals differences in the secondary structure between active ShlA242 and the three inactive derivatives (Walker *et al*., 2004). These results clearly demonstrate that activation of ShlA242 occurs through changes in conformation. Since ShlA242 secretion and activation reflect ShlA secretion and activation, and ShlA242 complements inactive ShlA and ShlAΔ3–97, the conclusions drawn for ShlA242 apply for ShlA. The secretion and activation domain of ShlA is located in the N-terminal segment of approximately 240 residues. This finding presumably applies also to the other cytolysins of the *S. marcescens* type, and this assumption is supported by the cross-complementation of non-hemolytic HpmA of *P. mirabilis* by ShlA269 and of inactive ShlA by an inactive N-terminal HpmA fragment, and activation of non-hemolytic HpmA by ShlB, despite the lack of cross-reaction of a polyclonal anti-ShlA antiserum with HpmA.

## SECRETION OF ShlA

The elucidation of the mechanism of ShlA secretion by ShlB across the outer membrane was first approached by studying the transmembrane topology of ShlB. All outer membrane proteins with determined crystal structures consist of antiparallel β-strands connected by surface loops of various length and of short periplasmic turns. The β-strands form β-barrels that are partially or completely closed by surface loops, globular domains, or an α-helix that inserts into the β-barrels from the periplasmic side. It is likely that ShlB has the same basic design. The antigenic 11-residue M2 epitope has been inserted at different sites along the ShlB protein, and the reaction with a monoclonal antibody has been determined in cells (Könninger *et al*., 1999). Since the antibody does not enter the cells, only those epitopes exposed to the cell surface react, thereby identifying amino acid residues of surface loops. These data have been compared with data obtained with isolated outer membranes, leading to the determination of additional sites in transmembrane areas or in periplasmic turns. A tentative model of ShlB that predicts 20 transmembrane β-strands has been derived (Könninger *et al*., 1999).

In another study, a tetrapeptide has been inserted at 11 sites of ShlB, which yielded, among others, two mutants that secrete inactive hemolysin (Yang *et al*., 2000). The inactive ShlA derivatives are activated *in vitro* by wild-type ShlB and complemented to active ShlA by ShlA255. The secretion-competent but activation-incompetent mutant with an insertion after residue 136 does not activate or secrete ShlA when the tetrapeptide is elongated to a 15-residue insertion by the M2 antigenic epitope (Könninger *et al*., 1999). Residue 136 is located in the most prominent predicted surface loop, and residue 224 is located in the adjacent transmembrane segment. In the two mutants, secretion is uncoupled from activation. With isolated and purified ShlB, activation is uncoupled from secretion. The two ShlB activities can be separated from each other, even though they might be coupled *in vivo*. The region defined by the two mutations seems to play a vital role in activation and a lesser role in secretion.

Secretion of ShlA by ShlB might basically occur as secretion of proteins by the autotransporter mechanism (type V secretion). Proteins secreted by the type V mechanism consist of two domains—the transporter and the passenger domains. The transporter domain integrates into the outer membrane and allows translocation of the passenger domain. Once at the cell surface, the passenger domain is autoproteolytically cleaved from the transporter domain and released from the cells, whereas the transporter domain remains in the outer membrane. In the case of ShlAB, the covalently linked domains of the autotransporters are separate and encoded by two genes. ShlB is equivalent to the transporter domain and ShlA to the passenger domain.

There are conflicting data on the structure of the transporter domain of autotransporters that have relevance for the export mechanism. The crystal structure of the transporter domain of the NalP exoprotease of *Neisseria meningitidis* reveals a 12-stranded β-barrel filled with an N-terminal α-helix (Oomen *et al*., 2004). This structure suggests that the proteolytic passenger

domain cleaves itself from the α-helix after translocation across the outer membrane through the channel formed by the β-barrel. After translocation of the passenger domain, the α-helix closes the channel, through which poisonous compounds could enter the cells. The diameter of the pore in the β-barrel allows passage of only an unfolded protein, in agreement with studies on the secretion of the IgA protease of *Neisseria gonorrhoeae* (Maurer *et al*., 1997), which is the first described example of an autotransporter. In contrast to the single NalP molecule, electron microscopy of the IgA translocator domain, overproduced in *E. coli*, reveals a multimeric structure (Veiga *et al*., 1999). In addition, a disulfide cross-linked scfv antibody chain linked to the IgA translocator domain is secreted with low efficiency, which suggests translocation of a folded protein through a channel formed by the multimer.

These and other conflicting data discussed in Oomen *et al*. (Oomen *et al*., 2004) have led to the proposal of an alternative translocation model. In this model, the passenger domains of autotransporters are translocated across the outer membrane through a pore formed by the Omp85 protein, rather than through a pore formed by the transporter domain. The transporter domain plays a role in the specific recognition of the cognate passenger domain. Omp85 is important for the assembly of outer membranes of Gram-negative bacteria (Voulhoux *et al*., 2003) and might be involved in the translocation of large hydrophilic surface loops of integral outer membrane proteins.

The question arises as to which of the three autotransporter models, if any, applies to the translocation of ShlA by ShlB. In four experimental approaches, ShlB monomers rather than multimers have been observed. ShlB solubilized with a Tris-EDTA-1% octylglucoside buffer from outer membranes of *E. coli* transformants that synthesized ShlB or ShlB ShlA migrates on SDS-polyacrylamide gels at 25, 37, 50, and 100°C as one band of 60 kDa, which corresponds to the molecular mass of monomeric ShlB (Könninger *et al*., 1999). There is no evidence for multimer formation, as observed with the porins that form trimers in the same experiments at temperatures up to 50°C. Electrophoresis of column-purified ShlB in the presence of octylglucoside on a native gel also reveals only the 60-kDa band. Chemical cross-linking experiments also do not yield multimer ShlB forms. After treatment of spheroplasts with formaldehyde and glutaraldehyde, only ShlB monomers are observed after SDS-PAGE. After treatment with *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide-*N*-hydroxysucciniimide-3-sulfonic acid, trace amounts of a 115-kDa compound appear, which could represent an ShlB dimer (Könninger, doctoral thesis, 1998). A functional assay also provides no evidence for multimer formation. If ShlB forms a multimer, overexpression of an inactive ShlB mutant protein together with an active wild-type ShlB should reduce ShlB wild-type activity (negative complementation). Co-expression of wild-type ShlB with the secretion-competent but activation-incompetent mutant ShlB136-ARSG or ShlB224PDLA results in the same hemolytic activity as obtained with cells expressing only wild-type ShlB (Yang *et al*., 2000). These results demonstrate an ShlB monomer and exclude functionally relevant amounts of stable ShlB multimers. They suggest a secretion model in which ShlA is translocated through a pore formed by the ShlB β-barrel, or the involvement of a protein that is equivalent to the Omp85 protein. However, a homology search reveals only one protein in *S. marcescens* with 23% identity to Omp85, only between residues 214 and 471 from a total of 797 residues. For comparison, the *E. coli* homologue displays 31% identity over the entire neisserial Opm85 length.

Pore formation of ShlB in artificial lipid bilayer membranes was studied with a highly purified sample solubilized in 1% octylglucoside (Könninger *et al*., 1999). ShlB forms very small pores with a single channel conductance of 10 pS, which only occasionally opens for a short time to a much higher level of 1.25 nS. The results reveal the potential of ShlB to form pores that are mostly closed. This result is not unexpected since cells cannot tolerate a large, permanently open channel in the outer membrane. Rather, one might expect that the pore only opens during ShlA secretion. Two ShlB deletion derivatives—one lacking a portion of the predicted first surface loop, the adjacent transmembrane segment, and part of the second loop; the other lacking most of the second loop—form more stable channels of 1.25 nS. A pore with 1.25 nS conductance could accommodate an unfolded protein. As described above, the high trypsin sensitivity of ShlA prior to its secretion points to an unfolded conformation. Folding at the cell surface could provide the energy for translocation. To elucidate the secretion mechanism of ShlA by ShlB, it will be important to determine the crystal structure of ShlB. Gentle purification procedures have been developed that may form the basis for obtaining crystallizable samples (Hertle *et al*., 1997; Sauter *et al*., 2003).

## THE TWO-PARTNER SECRETION (TPS) PATHWAY

Secretion of the hemolysins of the *Serratia* type and of the large family of adhesins of the FHA type has been collectively termed two-partner secretion (TPS) (Jacob-

Dubuisson *et al*., 2001). In the present review, the sequences are compared and possible secretion mechanisms are discussed.

Attention has been focused on the secretion and activation determinant of ShlA—the region covered by the N-terminal fragments of ShlA that are secreted and activated by ShlB. This domain contains two sites conserved in related proteins (Figure 31.1): AN(69)PNL and LAN(109)PNGIS. Replacement of N69 and N109 by isoleucine results in secretion- and activation-incompetent ShlA derivatives. Both mutant derivatives are highly trypsin-sensitive, which suggests lack of a native conformation. The corresponding mutation of N71 and N111 does not affect ShlA secretion and activation (Schoenherr *et al*., 1993). The inactive derivatives defined two important sites for ShlA secretion and activation. As more cytolysin sequences of the *Serratia* type were elucidated, it became apparent that the ANPN and the LANPNGIS(T) sequences are largely conserved. In fact, a much larger region around the second domain is nearly identical among the cytolysins. A third domain, ILNEVIG in ShlA, is contained in all cytolysins (Figure 31.2).

Studies on the secretion of the filamentous hemagglutinin FHA of *Bordetella pertussis* revealed a secretion mechanism very similar to that of ShlA (summarized in Jacob-Dubuisson *et al*., 2001). FHA contains an N-proximal sequence of approximately 300 residues that contain three sequence motifs similar to the three sequence motifs in the secretion domain of ShlA. FHA with the N-proximal fragment is secreted by the FhaC protein, which displays sequence similarities (18% identity) to the ShlB protein, is contained in the outer membrane, and forms pores with properties similar to those of ShlB in artificial lipid bilayer membranes (Jacob-Dubuisson *et al*., 1999). FhaC changes its structure when it secretes the N-proximal secretion domain (Guedin *et al*., 2000). Transmembrane topology models have been predicted for ShlB and FhaC using computer programs designed for analyzing crystal structures of outer membrane proteins, insertion of antigenic epitopes, and protease cleavage sites along the entire length of proteins (Guedin *et al*., 2000; Könninger *et al*., 1999). The models of the two proteins agree fairly well, except in the position of the N-terminus. A transmembrane segment predicted for ShlB is surface-exposed in FhaC. This results an odd number (19) of β-strands for FhaC, which has not yet been found in crystal structures of outer membrane proteins, instead of the 20 β-strands found in ShlB. There is experimental evidence for the positioning of the N-terminus of FhaC at the cell surface and only weak evidence for the positioning of the N-terminus of ShlB in the periplasm. However, if the basic design of the crystal structure of the translocation domain of the NaP protease applies also for ShlB and FhaC, the N-terminus of each protein would form an helix inserted into the β-barrel from the periplasm. The N-terminus of ShlB would bind the N-terminus of ShlA, move along with ShlA through the pore of the β-barrel, and close it after translocation of ShlA has been completed. The predicted ShlB model, but not the predicted FhaC model, can be reconciled with such a structure. The determination of the crystal structures of ShlB and FhaC will reveal which model is closer to reality.

The crystal structure of the N-terminal secretion domain of FHA (304 residues, designated Fha44) has been determined to 1.7 Å resolution (Clantin *et al*., 2004). It forms a right-handed parallel β-helix of nine complete turns that contains 37 parallel β-sheets (Figure 31.3). It is highly likely that the secretion domain of ShlA assumes a similar structure. The NPNL and ANPNGI motifs, indicated in Figure 31.3, form type I β-turns and are considered to stabilize the structure. Their role may be more than structural in that they contribute to recognition of ShlA (FHA) by ShlB (FhaC). The β-helix supports the theory that ShlA (FHA) is secreted as an extended structure that folds at the bacterial surface. The location of the highly conserved ILAEVT motif (Figure 31.2) is also shown in Figure 31.3.

A BLAST search reveals over 100 cell surface proteins, eight of which are hemolysins and the rest are predicted to be adhesins/hemagglutinins (Figures 31.1, 31.2). Besides the well-characterized FHA hemagglutinin of *B. pertussis*, secretion of the HMW (high-molecular-weight) adhesins of *Hemophilus influenzae* and the Lsp proteins of *Haemophilus ducreyi* have been studied in some detail. Two surface-exposed HMW proteins, HMW1 and HMW2, each require one protein, HMW1B/HMP2B and HMW1C/HMW2C, respectively, for secretion. Conversion of the NPNGI motif to IAIG results in loss of HMW1 secretion (St. Geme and Grass, 1998). In contrast to the secretion of a single protein by one export protein, secretion of the LspA1 and LspA2 proteins is mediated by one LspB protein, which is an orthologue of FhaC (Buscher *et al*., 2004).

## *ShlAB* AND ITS HOMOLOGUES: COMPARATIVE GENOME ANALYSIS

The comparison of the sequences of the ShlAB proteins deduced from sequenced genomes reveals that the N-terminal sequences of the ShlA proteins, which are recognized by ShlB proteins and their orthologs, exhibit a

```
ShlA            NQPGAVLNNSREA-GLSQLAGQLGANPNLGGR-EASVILNEVIGRNPSLLHGQQEIFGMAADYVLANPNGISCQSCGFINTSHSSLVVGNPLVENG-VLQGYSTFGNRNTL 182
EihA            NRPGAVFNNALTG-GNSQLAGALAANPNLHGQ-SASVILNEVISRNPSLLLGQQEVFGMAADYVLANPNGITCDGCGFINTTRSALVVGNPLVEDG-ALSAYSTLNNGNRL 181
EthA            NRPGAVFNNACTG-GESQLAGALAANPNLHGQ-SASVILNEVISRNPSLLLGQQEVFGMAADYVLANPNGITCDGCGFINTTRSALVVGNPLVE-GGALSAYSTLNNDNRL 181
PhlA            GQMGVVLNNGLEE-GQSQLAGQLAANPNLNGQ-AANLILNEVISRNPSLLLGQQEVFGIAADYVLANPNGITCNGCGFINTNRASLVVGNPLIEQG-NLQGFSTFNNQNSL 183
YhlA            GEKGAVLNNATSA-GNSILAGQLAANQNLNGQ-AASIILNEVISRNPSLLLGQQEIFGMTADYILANPNGITCNGCGFMNTNRESLVVGNPLIEQG-SLKGFETFNNNNWL 182
HpmA            GKPGAVFNNALEA-GQSQLAGHLNANSNLNGQ-AASLILNEVVSRNPLFLLGQQEVFGIAAEYVLSNPNGITCDGCGFINTSRSSLVVGNPLFENG-QLKGYSTLNNTNLL 152
Hly_Chrovi      NQPGAVFNNSLQG-GASQLAGQLAGNAMLGGN-QASVILNEVVSRNPSLLLGKQEVFGKAADYVLANPNGITCNGCGFINIPRASLLVGTANLQNG-AIASLQAANNGNAL 181
Adh_Borbro      ANPGVVFNNGLTD-GVSRIGGALTKNPNLTRQ--ASAILAEVTGTSPSRLAGTLEVYGKGADLIIANPNGISVNGLSTLNASNLTLTTGRPSVN-GG-RIGLDVQQGTVTI 169
FhaB_Borpe      ANPGVVFNNGLTD-GVSRIGGALTKNPNLTRQ--ASAILAEVTGTSPSRLAGTLEVYGKGADLIIANPNGISVNGLSTLNASNLTLTTGRPSVN-GG-RIGLDVQQGTVTI 218
FHA_Borpe       ANPGVVFNNGLTD-GVSRIGGALTKNPNLTRQ--ASAILAEVTDTSPSRLAGTLEVYGKGADLIIANPNGISVNGLSTLNASNLTLTTGRPSVN-GG-RIGLDVQQGTVTI 218
FhaS_Borpe      GPPGLVFNNSLTE-GRSQLAGYLNRNPGLRQA--ATAILTEITGTRPSRLHGALEVFGRRADLIVANPNGLSVDGLVTLNAGRLTLSTGRPVTE-GG-RLRLDVDAGHIAV 193
Adh_Pseufl      DRQGLVLNNALQA-GQSQLAGQLAANPQLQGQ-AASVILNEVISRNPSAINGAQEIFGRAADYVLANPNGISVNGGSFINTPNANLLVGRPELNDG-KLQALSTRDATGQL 186
Adh_Yerpe       DGRGVILNNGHG-VNQTELGGFIDGNPWLARG-EASIILNEVNSRDPSKLNGYIEVAGRKAQVVIANSAGITCEGCGFINANRVTLTTGQAQLNNG-LLTGYDVERGDIVI 226
HecA            DNRGLILNNGTARLTPSQLGGLIQNNPNLNGR-AAAAILNEVVSPNRSRLAGYLEVAGQAANVVVANPYGITCSGCGFLNTPRLTLTTGTPQFDAAGGLSGLDVRGGDILI 198
Adh_Pseuae      GANGLILNNATGKTQGTQLGGIILGNPNLKGQ-AAQVILNQVTGGNRSTLAGYTEVAGQSARVIVANPHGITCQGCGFINTPRATLTTGKPIMDGQ-RLERFQVDGGDIVV 182
Adh_Pseusy      ESQGVILNNSTNQTQSTQLGGIIVGNSNLRGT-AATTILNEVVGANASQLKGYTEVAGQAARVIVANPYGISCNGCGFINTPQVTLTTGKPVLDANGQLQRFNVQGGSISI 200
Hly_Barthen     GHVGVIWNNHAQEVGHSQLGGIMPGNPHLRFSGSAKVILNEVTSGKRSALNGPGEVFGKQADVIIANPNGISCDGCGFINTPHATLTTGVPEIDVSGFLKGFVVKGGEITF 205
Adh_Ralstso     DGIGLILNNSLAG-GGTLLGGNVGGNANLATSGPASTILTQVTGTDPIRINGTVEVFGTPASVIFSAPAGIYTQGAGFTNTPRVTLSSGTPQFLNGSGANVSFDQATAVGF 265
FhaB_Xancamp    GPNGLILNNSAQI-SKTELGGYVAGNNNLQRSGAASLILNEVTSAS-SRLQGYTEIAGAKAQLVIANPNGISCDGCGFLNTSRVTLTTGAPNLGSDGALNGFTVTGGALSI 191
Hly_Ecoli       DKNGVVFNNSANE-SSTSLAGNIQGNSNLT-SGSAKVILNEVTSKNPSTINGMMEVAGDKADLIIANPNGITVNGGGSINTGKLTLTTGTPDIQDD-KLAGYSVNGGTITL 180
HMW1_Haedu      PNSAVFNNSRTES--TSQLVGKLHANIQLQKE--AKLILNQVTGDHESNIQGALEVAGKKADLIIVNPNGITLNGVKTINTDRFVVSTSDIIPHREN--GLLSVRNGKVTI 258
HhdA            DVSGVVLNNAQTD-IHTQLAGNISANPHLTQA-SANIILNEVVSKDPSKLLGKQEIAGKIADYILVNPNGMSCDGCGFINISNASLVVGNPNITDGV-LKGYRIDGSHNIS 175
Adh_Xylfas      TPQGAILNNARTP-AQTHLAGTVQGNPWLATG-TAKIILNEVNSRTPSQLHGYLEVAGDRAQLIIANPSGITCNGCGVINASQFTLTTGTPVFNARGALDHYRVHGGAIQL 222
FhlA            DGRGAILNNSKDL-TNSQLGGLIYGNPNLQNSKEASTIINEVSGVNKSRIEGYQEIVGKRANYILANPNGIYINGAGFINTGNVTFTTGRGNNLLNP-------EKGMIEI 170
FloA            DFGLSLKNNAEA----------------------ELIINEVVSKDFSILRGELELQGKKATVVIANPNGISCRDCSFSGIDDIKLIAGSSTGKFSK-----------TF 147
Adh_Neime       DEKGVILNNSRSN-TQTQLGGWIQGNPHLARG-EARVIVNQIDSSNPSLLNGYIEVGGKRAEVVVANPSGIRVNGGGLINAASVTLTSGVPVLNNGN-LTGFDVSSGKVVI 232
Adh_Actpl       DSKGAILNNSRSQ-TQTQLAGMVQGNPNLSRG-EAKVILNEVNSSDPSILKGYIEVAGKKADVIIANPSGLHCEGCGVINADRATFTTAKPHIQNGS-LESFVVEKGKVKV 207
PfhB1           PNSAVFNNNGTEAQARSTLIGYIPQNQNLRGGKEADVILNQVTGPQESKIVGALEVLGKKADIVIANQNGITLNGVRTINSDRFVATTSELIDPNQM---MLKVTKGNVII 265
HMW1_Haein      NSVDAIINWKQFNIDQNEMVQFLQENNN------SAVFNRVTSNQISQLKGILDSNGQ---VFLINPNGITIGKDAIINTNGFTASTLDISNENIKARNFTFEQTKDKAL 194
                       *                          :. .: .    : *  :  *     ..    *:       .
```

**FIGURE 31.2** Alignment of the N-terminal secretion and activation domain of ShlA with related proteins. ShlA (hemolysin of *S. marcescens*), EihA (hemolysin of *E. ictaluri*), EthA (hemolysin of *E. tarda*), PhlA (hemolysin of *P. luminescens*), YhlA (putative hemolysin of *Y. pestis* CO92), HpmA (hemolysin of *P. mirabilis*), Hly_Chrovi (putative hemolysin of C. violacea), FhaC_Borbro (hemagglutinin C of *B. bronchiseptica*), FhaB_Borpe and FhaS (hemagglutinins of *B. pertussis*), AdhA_Pseufl (putative adhesion of *P. fluorescens*), AdhA_Yerpe (putative adhesin of *Y. pestis*), HecA_Erwchr (adhesin of *E. chrysanthemi*), AdhA_Pseuae (putative adhesin of *P. aeruginosa*), AdhA_Pseusy (putative adhesin of *P. syringae*), HecA_Barhen (adhesin of *B. henselae*), AdhA_Ralso (putative adhesin of *R. solanaceum*), FhaB_Xancam (adhesin of *X. campestris*), Hly_Ecoli (putative hemolysin of *E. coli*), HMW1_Haedu (high-molecular-weight protein of *H. ducreyi*), HhdA (hemolysin of *H. ducreyi*), Adh_Xylfas (putative adhesin of *X. fastidiosa*), FhlA (putative hemolysin/hemagglutinin of *F. nucleatum*), FloA (hemagglutinin of *P. mirabilis*), Adh_Neime (putative adhesion of *N. meningitidis*), Adh_Actsp (putative adhesin of *Acinetobacter* sp. strain ADP1), PfhB1 (adhesin of *P. multocida*), HMW1_Haein (high-molecular-weight protein of *H. influenza*) are shown.

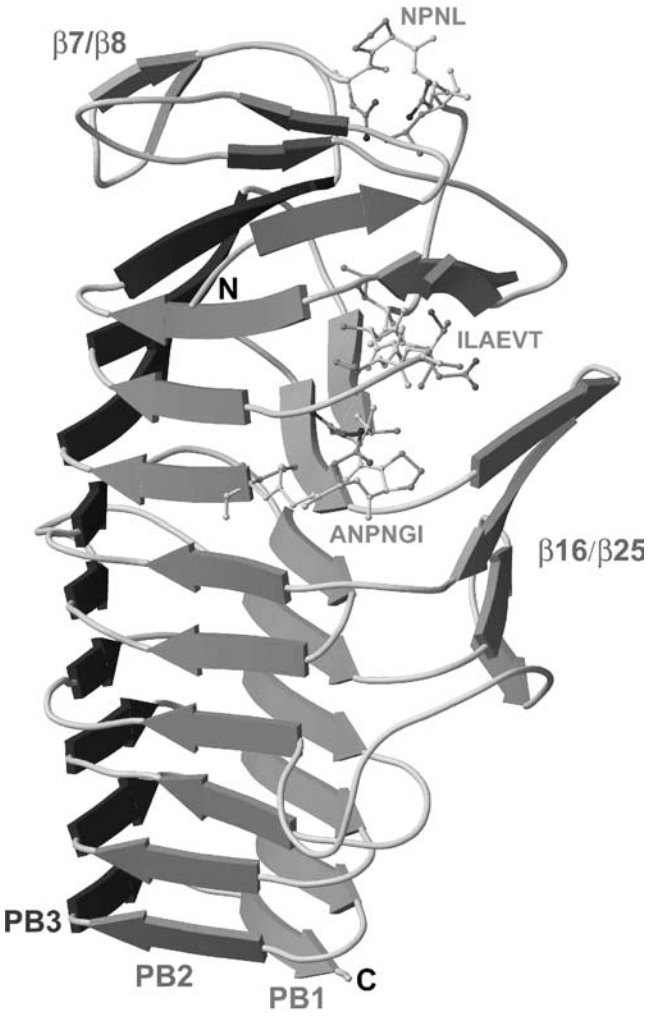



**FIGURE 31.3** Ribbon diagram of the crystal structure of the N-terminal region, residues 1–304 (Fha30), of the *B. pertussis* hemagglutinin. The regions NPNL, ANPNGI, and ILAEVT that are highly similar to regions of ShlA and related hemolysins and hemagglutinins are indicated in Figure 31.2. The structure consists of a right-handed parallel β-helix composed of three parallel β-sheets (PB1, PB2, and PB3), three extrahelical motifs, and an N-terminal capping. Reproduced with permission from Clantin *et al*., 2004.

much higher sequence identity than the entire hemolysins (in Figure 31.1A, only the sequences with high levels of identity are used for analysis). The sequence identity of the *H. ducreyi* ShlA protein is rather low, but it is a hemolysin (Palmer and Munson, 1995). The genomes of *Y. pestis* KIM and CO92 contain a second set of *shlA shlB* genes. There are currently 43 N-terminal sequences with 43–63% sequence identity to the ShlA N-terminus; most of the proteins with this N-terminal sequence are tentatively annotated as adhesins/hemagglutinins. In the ShlB family, there is a clear cut-off point between cytolysins and agglutinins at around 30% identity to ShlB.

In *S. marcescens* and *Y. pestis*, the *shlB-shlA* operons are located between the *lysC* (lysine-sensitive aspartokinase III) and *metH* (5-methyltetrahydrofolate-homocysteine methyltransferase) genes. Although the *lysC* and *metH* loci are close to each other in other organisms, such as *Erwinia carotovora, E. coli* O157 EDL933, *Salmonella typhi, P. luminescens,* and *P. mirabilis*, the distance between the two loci in *P. mirabilis* and *S. marcescens* is farther apart (1772 bp and 19065 bp, respectively), and the genes located between the two loci differ and contain no *shlAB* genes. The hemolysin genes of *P. mirabilis* and *P. luminescens* are located outside the *lysC-metH* locus.

## INTERACTION OF ShlA WITH MEMBRANES

In erythrocytes from various animal sources, ShlA showed no specificity for pore formation. This raised the question of why ShlA does not kill bacterial producer cells. Activation during or after secretion across the outer membrane by ShlB could be a means of protecting the bacterial producer cells. To test this hypothesis, stable protoplast-type L-forms of *P. mirabilis* that do not have an outer membrane were transformed with a plasmid carrying the *shlA shlB* genes (Sieben *et al*., 1998). Inactive ShlA* is secreted by the L-form cells, and ShlB is associated with the cytoplasmic membrane. The protoplasts are not lysed by ShlA, which suggests that the prokaryotic cytoplasmic membrane is resistant to ShlA. Some eukaryotic factors essential for pore formation are lacking in prokaryotic membranes. Specificity does not seem to be conferred by a protein receptor (Hertle, 2002). Rather, the lipid composition of the membrane is important for hemolysis. Negatively charged liposomes are amenable to ShlA-elicited lysis. The negative charge in eukaryotic membranes is mostly carried by phosphatidylserine (PS). Artificial liposomes with a composition reflecting that of erythrocytes—75% phosphatidylcholine (PC), 18% phosphatidylethanolamine (PE), and 7% PS—are lysed by ShlA. Liposomes consisting of 100% PC or 80% PC with 20% PE are completely resistant. *Serratia* and *Proteus* strains have up to 90% PE in the outer membrane and about 75% PE in the inner membrane, but lack PS, which renders these organisms resistant to ShlA treatment. The mechanism of pore formation is not well understood, but one can speculate that the negatively charged PS heads on the inside of eukaryotic membranes could play an essential role in the orientation and stabilization of the ShlA pore. Once bound to the membrane, active ShlA undergoes a conformational change upon membrane integration and

pore formation, whereas inactive ShlA (ShlA*) only binds to the membranes without any detectable conformational change (Hertle, 2002). These data also support the evidence of ShlA and ShlA* having different conformations.

## PORE FORMATION AND CYTOTOXICITY OF ShlA ON EUKARYOTIC CELLS

As *S. marcescens* is an important opportunistic pathogen that causes respiratory and urinary tract infections, bacteremia, endocarditis, keratitis, arthritis, and meningitis (Lyerly and Kreger, 1983; Maki *et al*., 1973), the role played by secreted and cell-bound ShlA is of interest. ShlA is not only a hemolysin, but also a cytolysin that damages tissues and causes release of the inflammatory mediators leucotrienes (LTB4 and LTC4) from leukocytes and the release of histamine from rat mast cells (König *et al*., 1987; Scheffer *et al*., 1988).

ShlA is inactive on keratinocytes, endothelial cells, and monocytes, but highly cytotoxic to epithelial cells, and elicits ATP depletion and potassium efflux in epithelial cells and in fibroblasts (Hertle *et al*., 1999). ATP is strongly depleted, down to 50% within minutes, even with sublytic doses of ShlA. Potassium efflux through the ShlA pores activates the membrane-bound Na/K-ATPase to restore the electrolyte levels. Pores formed by ShlA in the eukaryotic plasma membrane are smaller than the 1–3 nm estimated for ShlA pores in artificial black lipid membranes and in erythrocytes based on their failing to support the influx of propidium iodide ($M_r$ 668) and trypan blue, as measured by flow cytometry with cells depleted to 10% of the initial ATP level. ATP depletion is not affected by osmoprotection with oligosaccharides, which prevents ShlA-mediated hemolysis (Hertle *et al*., 1999). However, ATP depletion is reversible if vacuolation or cell lysis is prevented. Under these conditions, the ATP level is restored, presumably by the repair or closure of the ShlA-produced pores in the plasma membrane since repair requires protein synthesis, as evidenced by failure in the presence of cycloheximide.

The osmotic imbalance, indicated by potassium efflux, ATP depletion, and cell swelling, results in vacuolation. Small vacuoles appear all over the cytoplasm. Upon prolonged incubation, they fuse to form irregular larger vacuoles that fill the entire cytoplasm. Vacuolation is thought to be the result of a mechanism similar to macropinocytosis. Extracellular medium is found inside the spacious vacuoles. In contrast to vacuoles formed by the *Helicobacter pylori* cytotoxin VacA (Cover *et al*., 1993), vacuoles induced by ShlA are not acidified, vacuolation is not inhibited by bafilomycin, and vacuolation cannot be reversed, which indicates that the vacuoles are not derived from lysosomes.

## THE ROLE OF ShlA IN PATHOGENICITY

ShlA contributes to the uropathogenicity of *E. coli* 536/21, as shown with *shlA shlB* transformants (Marre *et al*., 1989). However, the pathogenicity of *Serratia* strains is a multifactorial process and includes the hemolysin, fimbriae, proteases, lipase, nuclease, and undefined determinants that facilitate invasion. These factors act in concert, and the resulting effects are adherence, host cell invasion, cytotoxic effects, and finally cytolysis. Because the hemolytic activity is mainly cell-associated, its effect arises predominantly after adherence of the bacteria to host cell tissues. The contribution of the hemolysin to *S. marcescens* pathogenicity has been shown with a *Caenorhabditis elegans* infection model, in which a hemolysin-negative mutant is less cytotoxic than the wild-type strain (Kurz *et al*., 2003).

Recently, it has been shown that ShlA in low doses induces apoptosis in Jurkat cells (Schallenberg and Hertle, unpublished observation). Hence, ShlA is a major factor in *S. marcescens* pathogenicity.

Invasion of tissue cells by *S. marcescens* depends on ShlA production (Hertle and Schwarz, 2004). Isogenic hemolytic-negative *S. marcescens* strains display a lower invasiveness than ShlA+ cells. The wild-type strain invades epithelial cells at a 0.10% entry level, whereas the hemolytic-negative mutant invades at a 0.0025% entry level. However, the cytotoxicity mediated by ShlA reduces the invasion of wild-type cells since ShlA cytotoxicity lyses the host cells, thereby decreasing the number of intracellular bacteria. In addition, adhesion of *S. marcescens* to the epithelial cells via mannose-sensitive pili (SafA) is essential for invasion (Hertle, Elsilk, and Priemer, unpublished observation). ShlA does not contribute to adhesion.

## CYTOTOXICITY OF ShlA-RELATED HEMOLYSINS/CYTOTOXINS

### HpmA of *Proteus mirabilis*

The *P. mirabilis* cytolysin HpmA acts as a potent cytotoxin against HRPTEC (human renal proximal tubular epithelial cells; Mobley *et al*., 1991), Daudi,

Raji (human-cell lymphoma), U973 (human monocytes), and Vero cells (African green monkey kidney cells) (Swihart and Welch, 1990). Cytotoxicity is determined as the release of lactate dehydrogenase (LDH); the cellular effects have not been studied in detail. As is the case with ShlA, cytolysis of target cells is very rapid and is caused by the pore formation by HpmA.

The ability of *P. mirabilis* to adhere to and invade urothelial cells is closely coupled to swarming (differentiation into hyperflagellated, filamentous swarm cells). Invasion is predominantly determined by flagellin production, and the hemolysin plays a minor role in this process (Allison *et al*., 1992). Invasion by swarm cells occurs within 30 min and is about 15-fold greater (about 0.18% entry after 2 h) than is invasion by vegetative cells (about 0.012% entry), which are internalized more slowly. A hemolysin-negative strain of WPM111 does not significantly alter virulence, but the flagella contribute to the ability of *P. mirabilis* to colonize the urinary tract, leading to acute pyelonephritis in an experimental model of ascending urinary tract infection. Inhibition of eukaryotic protein synthesis by cycloheximide does not reduce bacterial uptake. In addition, internalization of *P. mirabilis* into HRPTEC is decreased by HpmA because of cytolysis (Chippendale *et al*., 1994). The hemolysin-negative mutant strain WPM111 is recovered during the experiment in progressively higher numbers (10- to 100-fold) than the hemolytic parent strain BA6163.

### HhdA of *Haemophilus ducreyi*

*H. ducreyi* causes the sexually transmitted disease chancroid. It adheres to and penetrates epithelial cells (Lagergard *et al*., 1993) and is cytotoxic for HEp-2 and HeLa cells. Ulcer formation depends on iron deficiency (Sturm, 1997). Adherence is manifested after 15 min and reaches a maximum after 2–3 h. Cytotoxicity is ascribed to a cell-associated cytotoxin (Alfa, 1992), which is a hemolysin, HhdA, with sequence similarity to ShlA (Palmer and Munson, 1995). HhdA enhances the invasiveness of the organism and thus contributes to virulence (Wood *et al*., 1999). A screening of 90 *H. ducreyi* isolates has revealed the prevalence of the hemolysin genes *hhdB/hhdA*. The secreted hemolysin is immunogenic in a rabbit and primate infection model (Dutro *et al*., 1999).

Attachment to epithelial cells is mediated by more than one mechanism. Proteinase K treatment, but not trypsinization of *H. ducreyi*, significantly reduces attachment, which suggests the involvement of proteins. It is thought that the first step in the pathogenesis of chancroid is the adherence of bacteria to epithelial cells, followed by the action of cytotoxin and further bacterial proliferation. It has been suggested that this sequence of events results in the production of genital ulcers by *H. ducreyi*.

### EthA of *Edwardsiella tarda*

*E. tarda* can penetrate and replicate in HEp-2 cells (Janda *et al*., 1991). In contrast to other bacteria that replicate in the cytoplasm, such as *Listeria monocytogenes* and *Shigella flexneri*, *E. tarda* does not appear to harness actin or engage in direct cell-to-cell spreading. Invasion depends on the presence of EthA, a hemolysin of the ShlA type (Strauss *et al*., 1997). Hemolysin production enhances HEp-2 cell invasion, and a hemolysin-negative mutant strain (Tn*5* insertion in *ethB*) enters HEp-2 cells two or three orders of magnitude less efficiently than the parental strain.

### PhlA of *Photorhabdus luminescens*

*P. luminescens* is an entomopathogenic bacterium symbiotically associated with nematodes. Recently, it has been shown that hemolytic activity is iron-regulated (Brillard *et al*., 2002) and that this activity is due to secretion of PhlA mediated by PhlB. However, in a comparison of the wild-type strain to a chromosomal *phlA* mutant, no difference in virulence in an insect infection model was observed (Brillard *et al*., 2002) and the *phlBA* promoter was active. The authors hypothesize a possible cytolytic activity of PhlA against insect hemocytes and cells to convert insect cadaver tissue into a source of nutrients.

## CONCLUSION

The *S. marcescens* hemolysin forms the prototype of a number of pore-forming cytotoxins. The cytotoxic activities and related virulence-enhancing activities have been shown for the *S. marcescens* hemolysin and the hemolysins of *P. mirabilis, E. tarda, P. luminescens,* and *H. ducreyi*. The hemolysins identified from the analyses of sequenced bacterial genomes of *Y. pestis, Y. enterocolitica, C. violaceum, X. fastidiosa,* and *Edwardsiella ictaluri* remain to be characterized. Open questions concern the mechanisms of secretion and activation of the hemolysins. The hemolysins seem to be secreted like the adhesins of the *B. pertussis* hemagglutinin type. Data on the secretion of the hemolysins and the hemagglutinins might complement each other and result in an understanding of the novel secretion mechanism.

## REFERENCES


Alfa, M.J. (1992). Cytopathic effect of *Haemophilus ducreyi* for human foreskin cell culture. *J. Med. Microbiol*. **37,** 43–50.

Allison, C., Coleman, N., Jones, P.L. and Hughes, C. (1992). Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect. Immun*. **60,** 4740–4746.

Braun, V., Neuss, B., Ruan, Y., Schiebel, E., Schoffler, H. and Jander, G. (1987). Identification of the *Serratia marcescens* hemolysin determinant by cloning into *Escherichia coli. J. Bacteriol*. **169,** 2113–2120.

Brillard, J., Duchaud, E., Boemare, N., Kunst, F. and Givaudan, A. (2002). The PhlA hemolysin from the entomopathogenic bacterium *Photorhabdus luminescens* belongs to the two-partner secretion family of hemolysins. *J. Bacteriol*. **184,** 3871–3878.

Buscher, A.Z., Burmeister, K., Barenkamp, S.J. and Geme, J.W.S. (2004). Evolutionary and functional relationships among the nontypeable *Haemophilus influenzae* HMW family of adhesins. *J. Bacteriol*. **186,** 4209–4217.

Chippendale, G.R., Warren, J.W., Trifillis, A.L. and Mobley, H.L. (1994). Internalization of *Proteus mirabilis* by human renal epithelial cells. *Infect. Immun*. **62,** 3115–3121.

Clantin, B., Hodak, H., Willery, E., Locht, C., Jacob-Dubuisson, F. and Villeret, V. (2004). The crystal structure of filamentous hemagglutinin secretion domain and its implications for the two-partner secretion pathway. *PNAS USA* **101,** 6194–6199.

Cover, T.L., Reddy, L.Y. and Blaser, M.J. (1993). Effects of ATPase inhibitors on the response of HeLa cells to *Helicobacter pylori* vacuolating toxin. *Infect. Immun*. **61,** 1427–1431.

Dutro, S.M., Wood, G.E. and Totten, P.A. (1999). Prevalence of, antibody response to, and immunity induced by *Haemophilus ducreyi* hemolysin. *Infect. Immun*. **67,** 3317–3328.

Guedin, S., Willery, E., Tommassen, J., Fort, E., Drobecq, H., Locht, C. and Jacob-Dubuisson, F. (2000). Novel topological features of FhaC, the outer membrane transporter involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *J. Biol. Chem*. **275,** 30202–30210.

Hertle, R. (2002). *Serratia marcescens* hemolysin (ShlA) binds artificial membranes and forms pores in a receptor-independent manner. *J. Membrane Biol*. **189,** 1–14.

Hertle, R., Brutsche, S., Groeger, W., Hobbie, S., Koch, W., Könninger, U. and Braun, V. (1997). Specific phosphatidylethanolamine dependence of *Serratia marcescens* cytotoxin activity. *Mol. Microbiol*. **26,** 853–865.

Hertle, R., Hilger, M., Weingardt-Kocher, S. and Walev, I. (1999). Cytotoxic action of *Serratia marcescens* hemolysin on human epithelial cells. *Inf. Immun*. **67,** 817–825.

Hertle, R. and Schwarz, H. (2004). Internalization of *Serratia marcescens* in human epithelial cells is dependent on hemolysin. *BMC Infect. Dis*. **4,** 16.

Jacob-Dubuisson, F., El Hamel, C., Saint, N., Guedin, S., Willery, E., Molle, G. and Locht, C. (1999). Channel formation by FhaC, the outer membrane protein involved in the secretion of the *Bordetella pertussis* filamentous hemagglutinin. *J. Biol. Chem*. **274,** 37731–37735.

Jacob-Dubuisson, F., Locht, C. and Antoine, R. (2001). Two partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Mol. Microbiol*. **40,** 306–313.

Janda, J.M., Abbott, S.L. and Oshiro, L.S. (1991). Penetration and replication of *Edwardsiella* spp. in HEp-2 cells. *Infect. Immun*. **59,** 154–161.

König, W., Faltin, Y., Scheffer, J., Schöffler, H. and Braun, V. (1987). Role of cell-bound hemolysin as a pathogenicity factor for *Serratia* infections. *Infect. Immun*. **55,** 2554–2561.

Könninger, U.W., Hobbie, S., Benz, R. and Braun, V. (1999). The hemolysin-secreting ShlB protein of the outer membrane of *Serratia marcescens*: determination of surface-exposed residues and formation of ion-permeable pores by ShlB mutants in artificial lipid bilayer membranes. *Mol. Microbiol*. **32,** 1212–1225.

Kurz, C.L., Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G.P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J.A., Finlay, B.B., Gorvel, J.P., Ferrandon, D. and Ewbank, J.J. (2003). Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *EMBO J*. **22,** 1451–1460.

Lagergard, T., Purven, M. and Frisk, A. (1993). Evidence of *Haemophilus ducreyi* adherence to and cytotoxin destruction of human epithelial cells. *Microb. Pathog*. **14,** 417–431.

Lyerly, D.M. and Kreger, A.S. (1983). Importance of S*erratia* protease in the pathogenesis of experimental *Serratia marcescens* pneumonia. *Infect. Immun*. **40,** 113–119.

Maki, D.G., Hennekens, C.G., Phillips, C.W., Shaw, W.V. and Bennett, J.V. (1973). Nosocomial urinary tract infection with *Serratia marcescens*: an epidemiologic study. *J. Infect. Dis*. **128,** 579–587.

Marre, R., Hacker, J. and Braun, V. (1989). The cell-bound hemolysin of *Serratia marcescens* contributes to uropathogenicity. *Microbial. Pathogenesis*. **7,** 153–156.

Maurer, J., Jose, J. and Meyer, T.F. (1997). Autodisplay: One-component system for efficient surface display and release of soluble recombinant proteins from *Escherichia coli. J. Bacteriol*. **179,** 797–804.

Mobley, H.L., Chippendale, G.R., Swihart, K.G. and Welch, R.A. (1991). Cytotoxicity of the HpmA hemolysin and urease of *Proteus mirabilis* and *Proteus vulgaris* against cultured human renal proximal tubular epithelial cells. *Infect. Immun*. **59,** 2036–2042.

Oomen, C.J., van Ulsen, P., Van Gelder, P., Feijen, M., Tommassen, J. and Gros, P. (2004b). Structure of the translocator domain of a bacterial autotransporter. *EMBO J*. **23,** 1257–1266.

Palmer, K.-L. and Munson, R.-S.-J. (1995). Cloning and characterization of the genes encoding the hemolysin of *Haemophilus ducreyi. Mol. Microbiol*. **18,** 821–830.

Poole, K. and Braun, V. (1988). Iron regulation of *Serratia marcescens* hemolysin gene expression. *Infect. Immun*. **56,** 2967–2971.

Poole, K., Schiebel, E. and Braun, V. (1988). Molecular characterization of the hemolysin determinant of *Serratia marcescens. J. Bacteriol*. **170,** 3177–3188.

Sauter, S.R., Diekmann, S. and Braun, V. (2003). Two-step purification of the outer membrane transporter and activator protein ShlB from *Escherichia coli* using internally His(6)-tagged constructs. *J. Chrom. B Anal. Tech. Biomed. Life Scie*. **786,** 33–37.

Scheffer, J., Konig, W., Braun, V. and Goebel, W. (1988). Comparison of four hemolysin-producing organisms (*Escherichia coli, Serratia marcescens, Aeromonas hydrophila,* and *Listeria monocytogenes*) for release of inflammatory mediators from various cells. *J. Clin. Microbiol*. **26,** 544–551.

Schoenherr, R., Tsolis, R., Focareta, T. and Braun, V. (1993). Amino acid replacements in the *Serratia marcescens* hemolysin ShlA define sites involved in activation and secretion. *Mol. Microbiol*. **9,** 1229–1237.

Sieben, S., Hertle, R., Gumpert, J. and Braun, V. (1998). Expression and secretion of *Serratia marcescens* hemolysin in stable protoplast type L-forms of *Proteus mirabilis. Arch. Microbiol*. **170,** 236–242.

St.Geme, J.W.3. and Grass, S. (1998). Secretion of the *Haemophilus influenzae* HMW1 and HMW2 adhesins involves a periplasmic intermediate and requires the HMWB and HMWC proteins. *Mol. Microbiol*. **27,** 617–630.

Strauss, E.J., Ghori, N. and Falkow, S. (1997). An *Edwardsiella tarda* strain containing a mutation in a gene with homology to *shlB* and *hpmB* is defective for entry into epithelial cells in culture. *Infect. Immun*. **65,** 3924–3932.

Sturm, A.W. (1997). Iron and virulence of *Haemophilus ducreyi* in a primate model. *Sex. Transm. Dis*. **24,** 64–68.

Swihart, K.G. and Welch, R.A. (1990). The HpmA hemolysin is more common than HlyA among *Proteus* isolates. *Infect. Immun*. **58,** 1853–1860.

Veiga, E., de Lorenzo, V. and Fernandez, L.A. (1999). Probing secretion and translocation of a beta-autotransporter using a reporter single-chain Fv as a cognate passenger domain. *Mol. Microbiol*. **33,** 1232–1243.

Voulhoux, R., Bos, M.P., Geurtsen, J., Mols, M. and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299,** 262–265.

Walker, G., Hertle, R. and Braun, V. (2004). Activation of the *Serratia marcescens* hemolysin through a conformational change. *Infect. Immun*. **72,** 611–614.

Wood, G.E., Dutro, S.M. and Totten, P.A. (1999). Target cell range of *Haemophilus ducreyi* hemolysin and its involvement in invasion of human epithelial cells. *Infec. Immun*. **67,** 3740–3749.

Yang, F.-L., Hobbie, S. and Braun, V. (2000) ShlB mutants of *Serratia marcescens* allow uncoupling of activation and secretion of the ShlA hemolysin. *Int. J. Med. Microbiol*. **290,** 529–538.

CHAPTER

# 32

# Alpha-helix and beta-barrel pore-forming toxins (leucocidins, alpha-, gamma-, and delta-cytolysins) of *Staphylococcus aureus*

*Gilles Prévost, Lionel Mourey, Didier A. Colin, Henri Monteil, Mauro Dalla Serra, and Gianfranco Menestrina*

## INTRODUCTION

Among more than 40 peptidic toxins that can be secreted by *Staphylococcus aureus*, pore-forming toxins (PFT) constitute one of the most important groups besides staphylococcal superantigens (Prévost, 2005). This group of toxins is both diverse and multiple among those toxins secreted by this bacterium. This may bring an advantage towards the memory immune response that can be overcome by the simultaneous secretion of several antigenically related compounds bearing similar functions. Staphylococcal pore-forming toxins are divided into two subgroups comprised of those with alpha-helical structures and those rich in beta-strand sequences. Delta-hemolysin is one example of an alpha-helix structured toxin, while beta-sheet–rich PFT can be further divided into two subfamilies of a homo-oligomeric toxin, alpha-hemolysin and hetero-oligomeric bicomponent leucotoxins. Recent years have brought several structures' determinations to light, and further knowledge in their mechanism of action, roles in the pathogenicity by *in vivo* investigations, and molecular studies applied to intoxinated cells have also been the focus of numerous works. Perspectives of applications with beta-rich PFT are currently being explored. This review deals with the most recent findings with regards to these two groups of toxin.

## AN ALPHA-HELIX CYTOLYSIN PROTOTYPE: DELTA-HEMOLYSIN

The staphylococcal delta-hemolysin is 26 amino acid residues long, and forms a single amphiphilic α-helix that is supposed to associate in a lypical barrel-stave octamaric pore (Tappin *et al*., 1988; Dufourcq *et al*., 1999). This octamer has to cross the lipid bilayer to determine a pore by the space created by the monomers. Each monomer bears an amphipathic structure, which allows interaction with phospholipids while displaying a hydrophilic lumen inside the pore. Delta-hemolysin displays a very large blood cell spectrum and other cells, as well as membrane models, may also be lysed very actively. There is no evidence of pore formation into 1-palmitoyl, 1-2 oleoyl-sn-glycero-3-phosphocholine vesicles, whereas permeabilization of the vesicles concurrently occurs to the translocation of that toxin (Pokorny *et al*., 2002). In the latter case, formation of trimers was suggested. Some relatives of this toxin were characterized in other staphylococcal

species such as *Staphylococcus epidermidis*, *S. simulans*, *S. warneri*, and in bee venom. Delta-hemolysin is expressed at late exponential growth, and the corresponding encoded gene is included in that of the accessory gene regulator locus (Peng *et al*., 1988), whose expression depends essentially on the extracellular concentration of signal molecules, such as small peptides. The main question concerns the amounts of delta-toxin that a strain is able to produce while spreading to various cells. This level of expression, depending on that of a regulatory factor, may never be high. However, the lack of *agr* expression may lead to antibiotic resistance, but it is generally linked to a deficit in virulence (Sakoulas *et al*., 2002; Schwan *et al*., 2003), at least in some infections. To date, no investigation that only focused on the invalidation of the delta-hemolysin encoding gene has been reported.

## A BETA-BARREL PORE-FORMING TOXIN PROTOTYPE: ALPHA-HEMOLYSIN

### A mushroom-shaped structure of the pore

α-hemolysin or α-toxin (Smith and Price, 1938) is in fact not so much active upon human erythrocytes (HRBC) as the γ-hemolysin HlgA-HlgB is (Berheimer, 1974; Prévost *et al*., 1995). These two toxins are secreted by almost all *S. aureus* strains. Alpha-toxin harbors different hemolytic activities according to mammalian species and is particularly active on rabbit red blood cells (RRBC). This is a 293-amino-acid-long secreted protein that has an alkaline pH of 8.11, after the removal of the 26 residues' signal peptide (Gray and Kehoe, 1981). As other leukotoxins, it is secreted at the late exponential growth phase. But contrary to bicomponent leukotoxins, the secreted monomers may aggregate in solution or oligomerize as functional complexes in the presence of sodium deoxycholate (Bhakdi, 1981), independently of the temperature (4°–45°C) (Watanabe *et al*., 1987; Belmonte *et al*., 1986; Forti and Menestrina, 1989). This property was nicely exploited to determine the three-dimensional structure of the mushroom-shaped heptamer forming the pore in its lipostable form (Song *et al*., 1996). The heptameric oligomerization of α-toxin was confirmed by MALDI-TOF analysis of oligomers in solution and 2D-crystallization combined with atomic force microscopy (Vécsey-Sémjen, 1997; Gouaux *et al*., 1994; Malghani *et al*., 1999). However, hexamers can also be observed in certain phospholipid composition (Czajkowski *et al*., 1998). This mushroom structure has a height of 100 Å and a similar width at its cap domain that does not completely insert into the membrane (Figure 32.1). The stem is arranged as a β-barrel, which inserts into the membrane for about 50 Å. In fact, three protein domains can be distinguished in a given protomer extracted from the whole structure. The core of the protein (or Cap) gathers a β-sandwich of two to six antiparallel β-sheets that account for 55%

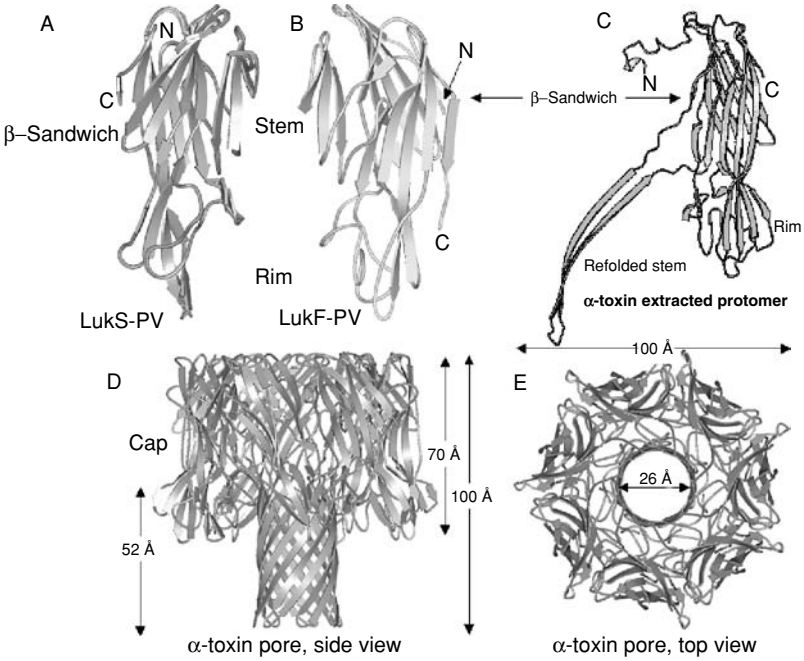



**FIGURE 32.1** Synthetic scheme of structures of LukS-PV (A), LukF-PV (B) with N- and C-terminal extremities mentioned, an α-toxin protomer (extracted from the structure of the oligomer) (C), the side view (D), and the top view (E) of the α-toxin.

of the total peptide sequence, and the rim that forms a four-stranded open-face β-sandwich and accounts for 28% of the peptide sequence. The third domain corresponds to the stem-central region that overlaps residues 109–150 and appears as a 65 Å long β-hairpin in the extracted protomer. The association of seven β-hairpins will form the β-barrel transmembrane pore whose diameter is 26 Å at its entry and which establishes after conformational changes consecutive to membrane interactions (Gouaux *et al*., 1997). The N-terminal extremity of the α-toxin is also extended and interacts with two adjacent monomers at the internal side of the pore mouth.

## Binding of α-toxin to membrane ligands

The interaction of α-toxin with RRBC was reported to be protease sensitive and consists of a high-affinity binding (Bhakdi and Tranum-Jensen, 1991), although it is able to form pores in pure lipid vesicles or may bind to some serum compounds at a lesser affinity (Bhakdi *et al*., 1983; Menestrina, 1986; Forti and Menestrina, 1989; Bhakdi and Tranum-Jensen, 1991). This toxin does not form a pore through the membrane of resistant cells, even if oligomerization may occur (Valeva *et al*., 1997). Moreover, it has a preference for synthetic bilayers constituted of choline-type phospholipids and cholesterol rather than other phospholipids (Freer *et al*., 1968) or sphingoglycolipids (Cheley *et al*., 1997). Recently, Pany *et al*. (2004) reported that residues from Trp179 to Trp187 of α-toxin bear a conserved pattern of binding to caveolin-1 from HyXHyXXXXHy/XXHy, where Hy is an aromatic residue. Purified caveolin-1 was able to dramatically reduce the α-toxin binding onto RRBC and its biological activity. Finally, a partition between high- and low-affinity membrane ligands cannot be excluded for such toxins because one of the intrinsic functions of PFTs is to interact with membranes.

## The α-toxin pore

When using synthetic membranes, the pore size and the conductivity of bulk solutions directly affect the conductance G that can be derived from the height of the current steps induced by the opening of pores: ($G = \sigma \,.\, \pi \,.\, r^2 \,/\, l$; where σ is the solution conductivity, and r and l are the radius and length of the pore, respectively). On the basis of electron microscopy data, the length of the pore was first estimated at 10 nm and the diameter at 1.1 nm (Menestrina, 1986), whereas a diameter of 2.7 nm was found using sugar exclusion methods (Krasilnikov *et al*., 1992; Bezrukov *et al*., 1996). In fact, the α-toxin pore is not a perfect cylinder, but rather a funnel with an entrance diameter of 2.8 nm, which decreases to a minimum diameter of 1.4 nm at the bottom of the stem (Song *et al*., 1996). Water molecules (Paula *et al*., 1999) and other non-electrolytes (Bashford *et al*., 1996) pass through these extrinsic pores, but the whole electrical field induced depends on charges at the entrance of the pores, which modify local concentrations for ions, and on secondary charges inside the course of the lumen, if any (Cescatti *et al*., 1991; Bezrukov *et al*., 1993). In the case of α-toxin, an anionic selectivity is essentially induced by charged residues that globally ensure an anionic filter: Asp45, Asn49, Lys51 (Song *et al*., 1996). In contrast, channels may select ions at their entrance and also inside their lumen (Doyle *et al*., 1998). This is not the case for pores formed by α-toxin. Therefore, the electrostatic filter provided by pores remains rather poor, but they well may be secondarily engineered. Conductance of pores may vary according to pH, applied voltage, or presence of divalent cations (Menestrina, 1986; Kasianowicz and Bezrukov, 1995; Menestrina and Vécsey-Sémjen, 1999). Although pore formation is quite an irreversible phenomenon, it is not sure whether or not the β-barrel could partially disassemble into a non-functional form because a low-conductance state representing 8% of the high-conductance was observed (Korchev *et al*., 1995a and b). Human fibroblasts have been observed to repair lesions caused by α-toxin (Valeva *et al*., 2000). This event was accompanied by a movement of residues oriented towards the lumen of the pore to a more lipophilic environment.

Besides residues involved in the interaction with membranes, some other residues like His35 form the interface between each monomer. Numerous substitutions of His35 have demonstrated its role in the oligomerization of α-toxin (Jursch *et al*., 1994; Menzies *et al*., 1994; Krishnasastry *et al*., 1994; Walker *et al*., 1995). Moreover, the concept of a prepore was demonstrated from the concurrent tests of oligomers' SDS-resistance and the modification of the environment of a single cysteine-substituting residue by using the environment-sensitive fluorophore acrylodan (Valeva *et al*., 1996). This prepore is in fact the result of multiple steps and a cooperative process in which the environment of residues located at the N-terminal extremity changes, as is the case for His35, for residues of the Rim, and especially for residues of the central domain (Valeva *et al*., 1996, 1997a, b, c). A periodicity of residues exposed either to the hydrophilic or to the hydrophobic compartments was found by using diazofluorene as a photoactivated probe (Lala and Raja, 1995). Residue 130 located at the extremity of the β-hairpin was demonstrated to be in a hydrophilic compartment by resonance energy transfer (Ward *et al*., 1994). Unfolding of the N-terminal extremity and of the stem domain are

associated and cooperative (Cheley *et al*., 1997). Interacting residues inside the oligomer were characterized by mutagenesis (Panchal and Bayley, 1995), and steps of the pore formation were tentatively arrested by artificial disulfide bond generation inside monomers (Kawate *et al*., 2003). These intricately conformational changes respond to a mechanism similar to the allosteric transition of an oligomeric enzyme, with the difference being that they are irreversible and cause minimal changes in the rest of the α-toxin structure (Valeva *et al*., 2001). The event mediating these concerted transitions remains to be defined.

## Target cells for α-toxin

Besides erythrocytes, α-toxin is active against a series of signaling cells such as T lymphocytes, platelets (Bhakdi *et al*., 1988), keratinocytes (Jonas *et al*., 1994; Walev *et al*., 1993), and fibroblasts (Walev *et al*. 1994), and other cells, such as PC12 (Fink *et al*., 1989), gastric (Thibodeau *et al*., 1994), chromaffin cells (Ahnert-Hilger *et al*., 1985), and endothelial lung cells (Seeger *et al*., 1990; Bhakdi *et al*., 1996). The activity of α-toxin onto rat aortic endothelial cells was shown to initiate a vasoconstriction through P-selectin, a surface protein also present in human PMNs, thus evoking that the interaction between the two kinds of cells is mediated by selectins (Buerke *et al*., 2002). IL-1 is secreted from α-toxin–treated monocytes, although these cells are not highly sensitive cells to lysis (Bhakdi *et al*., 1989). Alpha-toxin increases the arachidonic acid metabolism consecutive to the cell $Ca^{2+}$-activation, and generates prostacyclin, leukotriene secretions, and also nitric oxide from endothelial cells (Suttorp *et al*., 1985, 1987, 1993). Vascular leakage in perfused rabbit lungs was reported (Seeger *et al*., 1990). In a model of isolated perfused rat heart, α-toxin induced a liberation of thromboxane and prostacyclin that can be blocked by acetylsalicylic acid or indometacin (Sibelius *et al*., 2000). These features are associated with coronary vasoconstriction and loss of myocardial contractility, two symptoms that account for cardiac failures observed in cases of *S. aureus* septic shock. Simvastatin may act as an antagonist of the α-toxin–induced leucocyte-endothelial cell interaction (Pruefer *et al*., 2002). Similar cell inflammatory responses plus IL-8 expression were also confirmed in treated lung alveolar cells where NF-κB signal transduction seems to be involved (Rose *et al*., 2002).

## α-toxin and apoptosis

Some authors have suggested that apoptosis may be induced by α-toxin (Jonas *et al*., 1994), but the broad collection of data on that topic remains unclear. Such research can be aimed toward understanding what the cell effects of sublytic doses of toxin in the infection background are (Moreau *et al*., 1997). Thus, doses of toxin have to be thoroughly considered in these assays, as well as the purity and homogeneity of α-toxin. Granule-mediated cell killing by cytotoxic lymphocytes requires a functional membranolytic perforin and granule-associated serine proteases. Replacing perforin by α-toxin both in Jurkat and Yac-1 cells did not lead to apoptosis, probably meaning that pores were not effective for the entry of granzymes (Browne *et al*., 1999). The broad-spectrum caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, prevented necrosis, plasma membrane destruction, loss of mitochondrial membrane potential, IL-1 beta release (Bhakdi *et al*., 1999) and CD14 shedding induced by α-toxin in monocytic cells. This necrosis was concluded to be mediated by a cell death pathway through passive $K^+$ efflux and activation of caspase-like proteases (Warny *et al*., 1999). However, α-toxin was estimated to induce apoptosis in umbilical vein endothelial cells, since there was less DNA fragmentation induced by an α-toxin-deficient *S. aureus* mutated isolate (Menzies *et al*., 2000). *Staphylococcus aureus*-induced cell death and caspase activation are also mediated by α-toxin, and both events can be abrogated by specific antibodies and are not induced with supernatants of an α-toxin-deficient *S. aureus* strain (Bantel *et al*., 2001). The α-toxin induces caspase-8 activation in normal cells and in CD95-resistant Jurkat T cells lacking CD95, but not in cells stably expressing the anti-apoptotic protein Bcl-2, Fas-activated death domain. α-toxin induces the release of cytochrome C from intact cells and also from isolated mitochondria in a Bcl-2–controlled manner, thus demonstrating that *S. aureus* α-toxin triggers caspase activation via the intrinsic death pathway independently of death receptors. These findings suggested that a specific signaling pathway was used in *S. aureus*-induced cytotoxicity. Another study focused on mononuclear cells (MNC) evidenced that unlike Jurkat T cells, apoptosis was additionally mediated by a caspase-9-independent component (Haslinger *et al*., 2003). Monocytes, but not Jurkat T cells, produced the tumor necrosis factor TNF-alpha upon stimulation. Blocking that release with a TNF-alpha receptor antagonist partially decreased apoptosis. Thus, whereas in Jurkat T cells apoptosis is solely mediated by the mitochondrial pathway, in MNC endogenous TNF-alpha and a death receptor-dependent pathway seemed to be involved, which may contribute to the depletion of immune cells during an *S. aureus* infection. However, recent data obtained in Jurkat T cells suggest that α-toxin-induced cell death remains independent from caspase activation and internucleosomal DNA fragmentation and from their

respective inhibition (Essmann *et al*., 2003). Caspase inhibition had no effect on both the α-toxin-induced lactate dehydrogenase release and the ATP depletion. Electron microscopy demonstrated that in the presence of active caspases, α-toxin–treated cells displayed a necrotic morphology characterized by cell swelling and cytoplasmic vacuolation, and once again the α-toxin-induced cell death was suggested to operate in a caspase-independent, necrotic-like manner.

## STAPHYLOCOCCAL BICOMPONENT LEUKOTOXINS

Staphylococcal bicomponent leukotoxins (Woodin *et al*., 1959; Prévost *et al*., 1995c, 1999) represent a subgroup among PFT that operates via two distinct proteins, a class S (31 kDa) and a class F protein (34 kDa) that are necessary to create bipartite oligomeric pores into monocytes (M), macrophages (MΦ), and polymorphonuclear cells (PMN) membrane, which are common denominator targets (Table 32.1, Figure 32.2). The genes are tandemly arranged and co-transcribed: the gene encoding an S protein being upstream to that encoding an F protein and only separated by a thymine. But any class S protein may give a specific leukotoxin when combined with a class F protein (Szmigielski *et al*. 1998; Prévost, 1999). These temperature sensitive proteins are quite soluble (> 20 mg/mL), depending on pH and ionic strength, and they have basic isoelectric points comprised between 7.4 and 9.9 (Prévost, 1999). The S protein always binds first to membranes, then allowing the secondary interaction of an F protein before the oligomerization and the formation of the pores (Colin *et al*., 1994). Thus, proteins are cited in the same order, S then F, to evoke a leukotoxin. There is only limited sequence identity (< 25%) among S and F proteins and α-toxin, but these are much more homologous, especially into the central domain forming the pore (Figure 32.3). Homologues also include β-toxin from *Clostridium perfringens* (Hunter *et al*., 1993), and CytK and HlyII from *Bacillus cereus* (Baida *et al*., 1999; Lund *et al*., 2000; Menestrina *et al*., 2001), and the transmembrane domain of several other PFTs. There are several loci characterized, but any combinations of a class S protein with a class F protein may give a new leukotoxin. These loci are diversely encountered in clinical isolates and may be linked to different clinical diseases (Prévost *et al*., 2003). Before their mode of action is reviewed, these toxins will be individually considered.

### Panton-Valentine leucocidin (PVL)

PVL is formed by LukS-PV and LukF-PV (Woodin *et al*., 1960). Its secretion has been associated with furuncles (Cribier *et al*., 1992; Couppié *et al*., 1994). PVL-producing strains also may be isolated from respiratory tract infections (Prévost *et al*., 1995b; Couppié *et al*., 1997; Baba Moussa *et al*., 1999a; Lina *et al*., 2001; Gillet *et al*., 2002) with a "probable" greater frequency depending on sanitary conditions. In the past, occurrence of furuncles was

**TABLE 32.1** Diversity of the β-barrel-forming toxins from staphylococca and other bacteria.

| Bacterial species | Toxin – Locus accession number | Acronym | Nb aa | Reference |
|---|---|---|---|---|
| *Staphylococcus aureus* | α-hemolysin - M90536 | αHL | 293 | Gray *et al*., 1984 |
| | Panton-Valentine leucocidin, *luk-PV* - X72700 | LukS-PV | 286 | Prévost *et al*., 1995c |
| | | LukF-PV | 301 | |
| | γ-hemolysin, *hlg* -L01055 | HlgA | 281 | Cooney *et al*., 1993 |
| | | HlgB | 300 | Kamio *et al*., 1993 |
| | | HlgC | 286 | |
| | leucocidin R, *hlg* variant X64389 | LukS-R | 286 | Prévost *et al*., 1995c |
| | | LukF-R | 299 | |
| | leucocidin M-F-PV like, *lukMF'* -D42144, D83951 | LukM | 280 | Kaneko *et al*., 1997 |
| | | LukF'-PV | 296 | |
| | leucocidin E-D, *lukED* – Y13225 | LukE | 286 | Gravet *et al*., 1998 |
| | | LukD | 301 | |
| | LukE-LukD variant – BAB47174 | LukNS / LukNF | 311 327 | Morinaga *et al*., 2003 |
| *Staphylococcus intermedius* | leucocidin S/F-I, *lukI* – X79198 | LukS-I | 281 | Prévost *et al*., 1995a |
| | | LukF-I | 300 | |
| *Clostridium perfringens* | β-toxin, *cpb* -L13198 | Cpb | 309 | Hunter *et al*., 1993 |
| *Bacillus cereus* | hemolysin II, *hly*II-U94743 | Hly-II | 383 | Baida *et al*., 1999 |
| | cytotoxin K, *cyt* AJ277962 | CytK | 306 | Lund *et al*., 2000 |

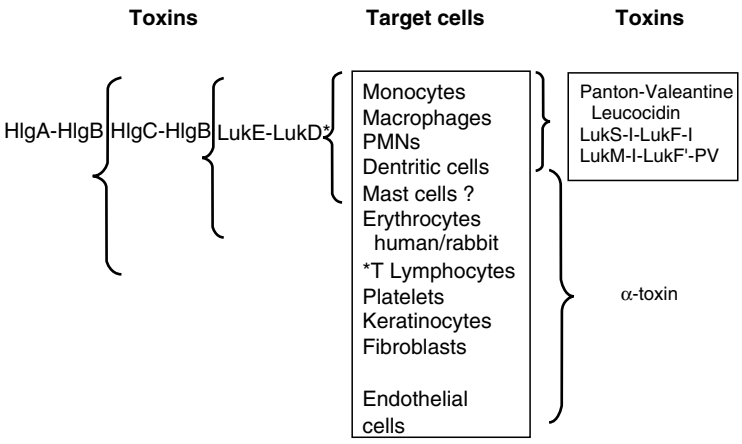



**FIGURE 32.2** Target cell spectra of the staphylococcal β-barrel pore-forming toxins. * LukE-LukD has a biological activity against T lymphocytes.

known to potentially evolve into septicemia, and bullous pneumonia of young children was clinically linked with frequent furuncles in patients or in their family. However, albeit rare, these community pneumonia are severe and often affect young adults as primitive or as secondary infections of influenza (van der Flier *et al*., 2003). The result of these infections is the necrosis of tissues and the eventual spreading of the bacteria. The occurrence of PVL-producing strains does not exceed 2–5% among human routine clinical isolates (Prévost *et al*., 1995b). The gene encoding PVL is located inside a bacteriophage (Kaneko *et al*., 1998; Narita *et al*., 2001). But the recent emergence of methicillin-resistant strains (MRSA) producing this toxin may confer an increased morbid risk and necessitates careful therapeutic attitudes (Vandenesch *et al*., 2003; Liassine *et al*., 2004). Such strains may be the result of the spreading of the PVL locus-carrying prophage after

```
                    : ...:: .:: .      .    . .. :   .. ..
LukF-PV        109 QVQQTVGYSYGGDINI----SNG-LSGGGNGSKSFSETIN  143
LukF-PV-like   109 QVQQTVGYSYGGDINI----ING-LSGGGNGSKSFSETIN  143
LukD           109 QVQQTLGYSYGGDINI----SNG-LSGGLNGSKSFSETIN  143
LukF-I         109 QVQNTLGYSFGGDISI----SKG-LSGGLNGSESFSETIN  143
HlgB           109 QVQNTLGYTFGGDISI----SNG-LSGGLNGNTAFSETIN  143
CytK           115 TVTSSVSYQLGGSIKASVTPSGPSGESGATGQVTWSDSVS  154
HlyII          103 KVTSEVGYTLGGSVKVGVNDKGPNADASITGSFAWKESVS  142
Hla            111 EYMSTLTYGFNGNVTG---DDTGKIGGLIGANVSIGHTLK  147
LukE           109 DVGQTLGYNIGGNFQ-----SAP--SIGGNGSFNYSKTIS  141
LukM           104 DVGQTLGYNIGGKFQ-----SVP--SIGGNGSFNYSKSIK  136
LukS-I         105 DVSQTLGYNVGGNFQ-----SAP--LLGGKGEFNYSKKIS  137
LukS-PV        104 NVSQTLGYNIGGNFN-----SGP--STGGNGSFNYSKTIS  136
HlgC           106 NVSQTLGYNIGGNFQ-----SAP--SLGGNGSFNYSKSIS  138
HlgA           104 DVSQKLGYNIGGNFQ-----SAP--SIGGSGSFNYSKTIS  136
Cpb            129 TVSNTMGYKIGGSIEI--EENKP--KASIESEYAESSTIE  164
                   ------------------>  ------------------>

PA             296 SRTHTSEVHGNAEVHA----SF  FDIGGSVSAGFSNSNS  329
Aerolysin      236 SEKVTTKNKFKWPLVG----ETE-LSIEIAANQSWASQNG  270
α-tox Clost.   170 GEKIGVKTSFKVGKEAIADSKVET-SFEFNAEQGWSNTNS  208
Cytotox Pseud. 104 KVGVEVKVKANIPLVG   GAEITSTVELSLSSTQGASTS  141
```

**FIGURE 32.3** Peptide alignment of the central stem domains that generate pores in short β-strand, rich pore-forming toxins and in other relatives: protective antigen (PA) of the anthrax toxin (Petosa *et al*., 1997), aerolysin from *Aeromonas hydrophila* (Parker *et al*., 1994), α-toxin from *Clostridium septicum* (Melton *et al*., 2004), and the *Pseudomonas aeruginosa* cytotoxin (Hayashi *et al*., 1989).

the removal of insertion sequences inside its genome, or of the mutation of the penicillin-binding protein 2a. PVL has a narrow specificity towards M, MΦ, and PMNs of humans and rabbits (Figure 32.2). In fact, it is active upon metamyelocyte and its lineage (Meunier *et al*., 1995). When interdermally injected in the rabbit skin, PVL is responsible for dermonecrotic lesions (Ward and Turner, 1980).

## Gamma-hemolysin

This ancient name that originates with a hemolytic activity operating on sheep, rabbit, horse, and human erythrocytes in fact gathers two distinct toxins, HlgA-HlgB and HlgC-HlgB (Smith and Price, 1938; Bernheimer, 1994; Guyonnet and Plommet, 1968; Cooney *et al*., 1993; Kamio *et al*., 1993; Prévost *et al*., 1995c). The first one has a broad range of target cells, including human T lymphocytes and erythrocytes (Figure 32.2), while HlgC-HlgB does not lyse T lymphocytes and is less active onto human erythrocytes (Prévost *et al*., 2001; Gauduchon *et al*., 2001). Of all known leukotoxins, HlgA-HlgB has the most potent efficacy on synthetic bilayers (PC:Cho, 1:1) (Ferreras *et al*., 1998). That toxin is secreted by almost all isolates, and an *hlg* deficient strain was shown to induce a slower growth, thus lowering inflammation in a rabbit endophtalmitis model (Supersac *et al*., 1996).

## LukE-LukD leukotoxin

This toxin was previously identified in the Newman strain (NTCC 8178) because of a residual leukotoxic activity in the culture supernatant of a *hla::tet, hlg::erm* double mutant, which does not secrete PVL (Gravet *et al*., 1998). It is located between a restriction-modification system *hsdS-hsdM* (Kuroda *et al*., 2001) and the hemine (*hem*) operon (von Eiff *et al*., 1997). It is produced by about one-third of the isolates, and a variant was characterized (Morinaga *et al*., 2003). In fact, more than 70% of isolates carry the corresponding encoding locus, and because of numerous mutations rendering these genes non-functional, this toxin should not be detected by genotyping tests (G. Prévost, personal communication). The production of LukE-LukD may occur both in methicillin-sensitive and -resistant strains. It is active upon human T lymphocytes in addition to PMN. The secreting isolates may be issued with a great probability from staphylococcal bullous impetigo where epidermolysin is also produced (Gravet *et al*., 2001) and from *S. aureus* antibiotic-associated diarrhea where enterotoxin (EntA > EntC > EntD) production also occurs (Gravet *et al*., 1999). These diarrhea affect aged patients (>65 years old) having undergone an antimicrobial therapy, often comprising a second-generation fluoroquinolone. LukE-LukD might enhance inflammation, at least in the tissues concerned by clinical associations. A bad prognosis can be encountered to the latter diarrhea with a significant risk of bacteremia, but an anti-staphylococci dedicated treatment can eradicate the infection, as well as stopping the treatment when possible. When injected in ligated ileal loops of rabbits, enterotoxin A and LukE-LukD generate a huge accumulation of bloody fluid inside the loop within 12 hours, which is associated with the degradation of intestinal microvillosities (Gravet, 2001).

## LukM-LukF-PV like

This leukotoxin has only been reported to be produced by animal strains isolated from bovine, ewe, and goat mastitis (Kaneko *et al*. 1997). It is distinct from PVL antigenically and by its biological activity (Gravet *et al*., 1998; Rainard *et al*., 2003). Its encoding operon is located onto a specific bacteriophage genome (Zou *et al*., 2000).

## LukS-I-LukF-I

The gene encoding LukS-I-LukF-I is expressed in probably all *S. intermedius* isolates (Prévost *et al*., 1995a). The presence of such a toxin into another bacterial species reveals the possible transfer and evolution of the genetic information for these toxins. That leukotoxin is active upon PMN and dendritic cells. *S. intermedius* is considered as a skin pathogen for small carnivores.

## Three-dimensional structures of soluble leukotoxin monomers

The structures of HlgB (Olson *et al*., 1999; PDB: 1LKF), LukF-PV (Pédelacq *et al*., 1999; PDB: 1PVL), and LukS-PV (Guillet *et al*., 2004a and b; PDB: 1T5R) have been successively determined by x-ray crystallography (Figure 23.1). These structures evidence the relation between bicomponent leukotoxins and α-toxin (Song *et al*., 1996; PDB: 7AHL), favoring the hypothesis of a common ancestor, with some differences probably sustaining the specifications of all these toxins. Compared to the α-toxin structure, those of the leukotoxin component revealed the necessary conformational modification of their central domains, folded as three β-strands at the hydrosoluble state into a long protruding, lipid-stabilized β-hairpin. The fold of the pore-forming leukotoxins superfamily shows a high content of β-strands defining a class of structurally related toxins with an ellipsoid shape. The largest domain gathers more than half of the sequences in two

six β-stranded antiparallel β-sheets that, after association of monomers, will form part of the cap domain of the mushroom-shaped heptameric pore (Song *et al*. 1996). The rim domain of these proteins that forms the bottom of the cap is composed of a four-stranded open-face β-sandwich, two helical structures, and two omega-loops whose arrangement varies according to the proteins. The three-dimensional structures of the highly homologous class F proteins, LukF-PV and HlgB (72% sequence identity), are similar, and only connecting loops of the rim and at the COOH-terminus display minor differences due to sequence variability. For a complete superimposition of the core and the rim domains of HlgB (or LukF-PV) to those of α-toxin, a rotation of about 10 degrees is necessary. The rim domains with exposed loops structures interact with the membrane, and some amino acids may be anchored in a lipid environment (Valeva *et al*., 1997a; Vécsey-Sémjen *et al*., 1997). It is evident that the organization of the rim domains in all known three-dimensional structures is not identical (see Figure 32.4).

## Binding properties of the bicomponent leukotoxins

In the circulating blood, it is not excluded that leukotoxins bind to soluble proteins, despite having low affinity (Katsumi *et al*., 1999). α-toxin and HlgB were co-crystallized with PC and LukF-PV with MES buffer, which shares part of its molecular skeleton with PC. In the structures of LukF-PV in HlgB and α-toxin, the conserved amino acids Trp176, Arg198, Tyr179, Glu191, and Arg197 delineate a crevice where Trp176 and Arg197 interact with PC (Figure 32.4). However, Pany *et al*. (2004) recently showed the interaction of caveolin-1 with a peptide motif of α-toxin including aromatic residues HyXXHyXXXXHy, which encompasses the first two amino acids of this binding site, and a dual binding in these proteins remains questionable. Caveolin-1 is normally absent at the trans side of human PMNs.

Phorbol myristyl-acetate positively activates protein kinase C pathway. Gauduchon *et al*. (2001) evidenced that this molecule negatively affects the binding of LukS-PV to humans PMN, whereas its inhibitor staurosporine restores this binding. The number of binding sites is about 197,000 for LukS-PV with an apparent $K_d$ around 0.07 nM, and an apparent $K_d$ for LukF-PV approaching 2 nM. LukS-PV and HlgC may compete at the same binding site, but not with HlgA or LukE. However, HlgA-HlgB is the leukotoxin having the greatest activity on synthetic bilayers (Meunier *et al*., 1997). Thus, these observations suggest that binding of at least some leukotoxins can be influenced by the presence of some receptors at the cell surface.

## Prepore-pore formation and calcium entry

Rapidly after the application of leukotoxins to human PMN and with the presence of physiological concentrations of $Ca^{2+}$, $Ca^{2+}$ enters the cells and gives rise to fluorescent probes (Fink-Barbançon *et al*., 1993; Colin *et al*., 1994). This entry of $Ca^{2+}$ has been shown to be inhibited by ethylene-glycol tetra-acetate, while the entry of ethidium is maintained. Moreover, drugs such as econazole or $La^{3+}$ also selectively inhibit this $Ca^{2+}$ influx (Staali *et al*., 1998). Other modifications of the stem domain, such as mutations or deletions, may result in the uncoupling of the two phenomenons (Baba Moussa *et al*., 1999; Werner *et al*., 2002). Finally, linking the central domain to the β-sandwich domain by two disulfide bridges in a state avoiding its unfolding into the β-hairpin abolishes pore formation and dramatically inhibits the entry of ethidium, but preserves that of $Ca^{2+}$ (Nguyen *et al*., 2002). Thus, the

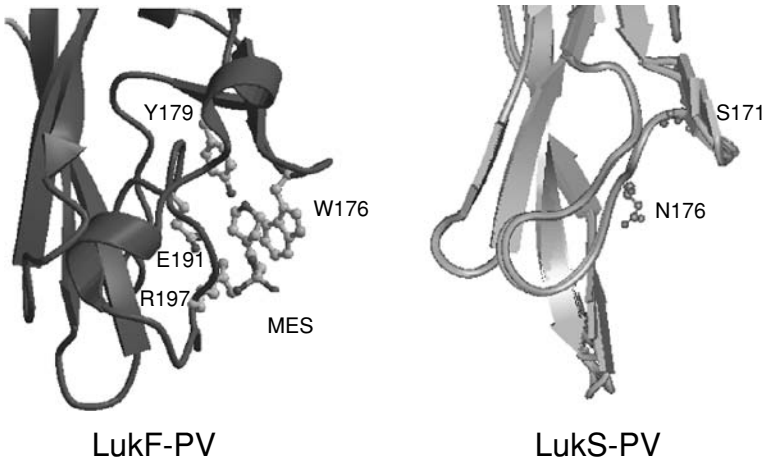



**FIGURE 32.4** Differences in the molecular architures of the rim domains of LukF-PV and LukS-PV. LukF-PV was co-crystallized with MES. HlgB and α-toxin were co-crystallized with phosphatidyl-choline (PC). MES and PC partially have similar molecular skeletons.

entry of $Ca^{2+}$ using other structures than the pores was strongly suggested.

The stoichiometry of the pore has been studied using different techniques, which leads to different results. First, kinetics of component associations strongly suggested an equal number of S and F protomes, and oligomers of an apparent molecular mass of 200 kDa were identified both onto PC-Cho small unilamellar vesicles and on PMN by SDS-PAGE on non-boiled samples (Ferreras *et al*., 1998; Werner *et al*., 2001). It has been shown that oligomers identified are immunologically reactive to both anti-S and anti-F antibodies. Therefore, due to the 4 kDa difference in the molecular mass of the two proteins, the formation of single protein homo-oligomers can be excluded. Moreover, Miles *et al*. (2002) constructed derivatives of HlgC and HlgB with C-terminal peptide tails that conserved the initial biological properties of leukotoxins. Then, non-boiled samples were applied to SDS-PAGE, and different ratios of the wild type toxins and mutants evidenced oligomers of different apparent masses, which were in favor of the formation octamers. Furthermore, the substitution into cysteine of residues located at the mouth of the lumen in each of the two proteins evidenced a four-step current modification when these residues reacted with the (2-sulfonaethyl) methane-thiosulfonate reagent, thus again suggesting octamers. However, Nguyen *et al*. (2003) suggested the formation of hexamers by using electron microscopy.

Moreover, Nguyen *et al*. (2002) showed that the combination of HlgA with an engineered HlgB, where the central domain was constrained to the protein core by a disulfide bridge, abolished pore formation but not the calcium entry, and that some kind of prepore was formed. Another fundamental difference in the structures of leukotoxin components (Pédelacq *et al*., 1999; Guillet *et al*., 2004b) with respect to that of α-toxin resides in the orientation of the N-terminal extremity. Whereas the N terminus is stacked to the upper part of the β-sandwich core in leucotoxins soluble monomers, it is deployed and interacts with two adjacent monomers at the entry of the pore lumen of the assembled α-toxin. Glutathion S-transferase fusion of S or F components at the N-termini keep significant biological activity. The sequence of the LukS-PV N-terminus remains seven residues shorter than that of LukF-PV. Thus, the interaction of the N-termini of bicomponent leukotoxins at the mouth of the lumen with adjacent monomers remains to be demonstrated. Also, the mode of accumulation of each type of monomer remains to be determined:

- Does the pre-location of several class S proteins determine the further interaction of class F proteins?
- Is the binding of one class S protein sufficient to assess further addition of S and F proteins?

## Bicomponent leucotoxins pore

After the assembly of monomers, the three-stranded central domains will unfold and edify the β-barrel transmembrane pore by a yet undefined mechanism. Pore formation is delayed to the activation and involves both class S and class F proteins. The diameter of the pore was suggested to be 19–21 Å by Sugawara *et al*. (1997, 2002) by using electron microscopy, while Baba Moussa *et al*. (1999) found a similar value by evaluating the exclusion of polyethylene glycols from the HlgA-HlgB pore, since molecules larger than 1,000 Da were excluded. Osmotic protection performed either with polyethylene glycols or sugars gave different values; they were homogenous when comparing the different bicomponent leukotoxins (Menestrina *et al*., 2003). Values for leukotoxins are about 10% higher than those for α-toxin. This may be due to a difference in the stoichiometry or to differently distributed charges inside the pore lumen. Leukotoxin pores are more sensitive to monovalent cations, and their opening disrupts the $Na^+/K^+$ gradient from the *trans* and *cis* sides of the membranes. Miles *et al*. (2001) showed that the conductance of the HlgC-HlgB pore was higher than that of α-toxin (190 pS vs 90 pS in 0.1 M NaCl, and 2.5 nS vs 0.775 nS in 1M NaCl), indicating divergences between these pores. Indeed, the selectivity of the α-toxin pore is rather anionic (Figure 32.5). Differences of charges pointing inside the pore lumen and the effect of their combinations have been emphasized by a mutagenesis approach (Comai *et al*., 2002). Whereas positive charges are found at the mouth of the α-toxin pore (Asp45, Asn49, Lys51), they are negative for leukotoxins (Asp43, Asp47, or Asn, Asp49). Indeed, when including positive charges at the mouth of HlgA-HlgB, selectivity became comparable to α-toxin. Other differences are also encountered in the pore lumen where negative charges are found in bicomponent leucotoxins that may not be compensated in the case of the HlgC-HlgB pore. By residue substitution, it has been possible to generate a HlgA-HlgB pore with a conductance selectivity similar to that of HlgC-HlgB.

## Bicomponent leukotoxins and cell response

Staphylococcal leukotoxins have been suggested to induce numerous cell responses that may be related with the $Ca^{2+}$ activation (Wang *et al*., 1990). They are able to induce leukotriene B4 and IL-8 secretion (Hensler *et al*., 1994a) and their expression (König *et al*., 1994). These two molecules are involved in leukocytes' recruitment (Figure 32.5). Histamine, which is respon-

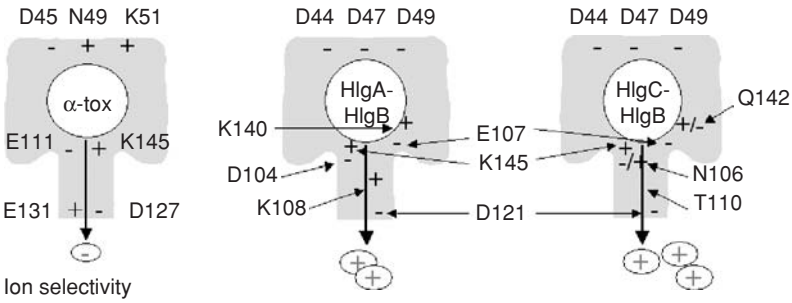



**FIGURE 32.5** Schematic representations of the pores formed by α-toxin, HlgA-HlgB, and HlgC-HlgB the hemolysins with charged residues solely or not that point inside the pore lumen, and the ionic selectivities associated.

sible for vasodilatation, is also produced (König *et al*., 1995). Control of the oxydative burst from human PMNs is also involved in inflammation (Colin *et al*., 2003) (Figure 32.6). Numerous cell pathways leading to IL-6, IL-12 secretion, and enzymes' secretion were shown to be involved (König *et al*., 1995, 1997; Prévost *et al*., 2003), but variations exist according to leukotoxins (König *et al*., 1997). Small GTP-binding proteins (Hensler *et al*., 1994b) were suggested to take part in cell activation, and leukotoxins might also induce apoptosis (Köller *et al*., 1993). Of course, cell lysis may, after activation, enhance mediators' liberation. These features are consistent with leukocyte recruitment, vasodilatation, and cell invasion and tissue necrosis observed in furuncles (Cribier *et al*., 1992) and in *S. aureus* community-acquired pneumonia (Couppié *et al*., 1997). The intradermal injection of PVL resulted in necrotizing lesions in rabbit skin (Cribier *et al*., 1992), but not in mouse skin, which suggests that the murine model may not be suitable.

## STAPHYLOCOCCAL PORE-FORMING TOXINS CHALLENGED IN PATHOGENESIS

Mutated strains with an α-toxin allelic replacement demonstrated the probable importance of this toxin in the course of infections, particularly in peritonitis (O'Reilly *et al*., 1986; Patel *et al*., 1987), mastitis (Bramley *et al*., 1989), arthritis (Nilsson *et al*., 1999),

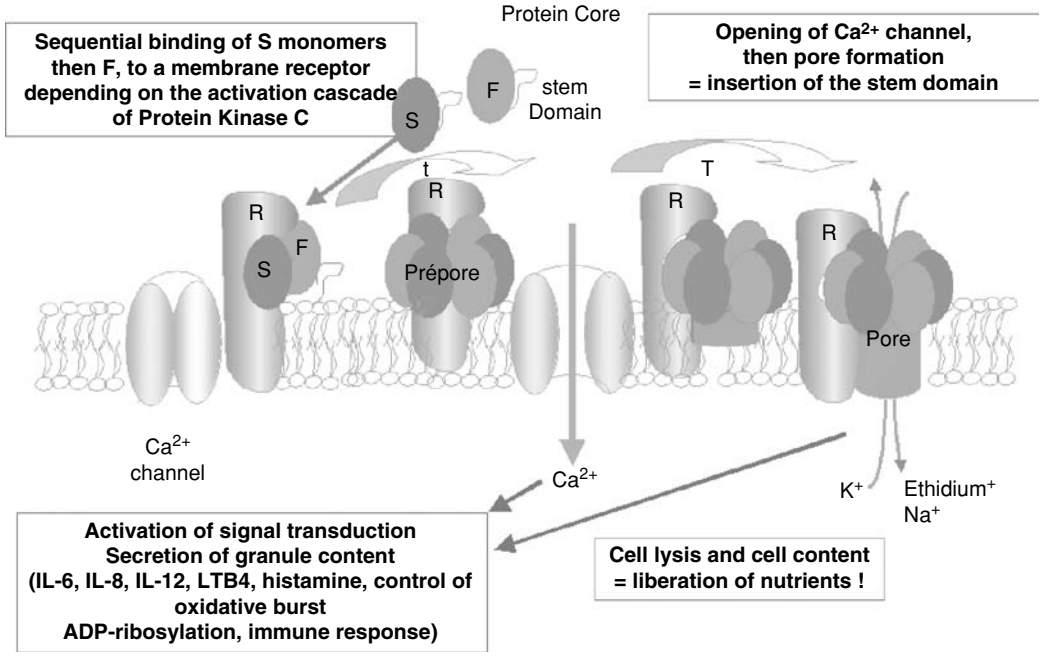



**FIGURE 32.6** Synthetic scheme of the mode of action of the staphylococcal bicomponent leucotoxins

corneal keratitis (O'Callaghan *et al*., 2003), and pneumonia (McElroy, 1999). Alpha-toxin is able to originate corneal disease in rabbits (O'Callaghan *et al*., 1997), and immunization against α-toxin reduced this damage, despite keeping the bacteria alive (Hume *et al*., 2000). Mutated Newman strains (NTCC 8178) for α-toxin or gamma-hemolysin genes produced infections with less dramatic lesions than those observed with the wild-type strain (Dajcs *et al*., 2002a). Finally, a deeper comparison between isogenic strains suggested that α-toxin and to a lesser extent γ–hemolysin may play a major role in the tissue destruction observed in keratitis (Dajcs *et al*., 2002b). These studies were focused on toxins whose genes were conserved in the *S. aureus* genome. Siqueira *et al.* (1997) compared the pro-inflammatory potential of bipartite leucotoxins in the posterior chamber of the rabbit eye. They observed that these toxins induce a considerable inflammatory reaction that may extend toward the anterior chamber and the entire eyeball, depending on the dose. In fact, the different S and F components can be combined to generate more leukotoxins than those which could be represented by the encoding genes. These combinations may generate a disease simulating endophthalmitis. This phenomenon might provide the advantage of escaping the humoral immune defense. When injected in ligated ileal loops of rabbits, enterotoxin A and LukE-LukD generate within 12 hours a huge accumulation of bloody fluid inside the loop associated with the degradation of intestinal microvillosities (Gravet, PhD thesis, 2001). In another approach, Schwan *et al*. (2003) isolated from a signature-tagged mutagenesis library *agr*C and *agr*A (assessory gene regulators C and A) defective mutants that allowed similar survival in a model of murine abscess. Moreover, an α-toxin defective mutant induced the same survival level as for the two *agr* genes' mutants. The *agr* mutated strains were not hemolytic, and most of the exoproteins were repressed. These authors suggested a potential role of α- and δ-toxins contributing to wound progress. However, no statement was developed about γ-hemolysin, which are hemolytic for a wide series of mammals and whose expression remains sensitive to *agr* loss. Often, the toxin genotype of the considered isolates remains unknown, some isolates may directly be issued from clinics, and the role of other virulence factors is difficult to exclude. Besides these observations, few authors checked for the effective expression of α-toxin or other virulence factors in the conditions of an infection where the inoculum was not huge. In a *S. aureus* infective endophthalmitis model, leucotoxins were shown to be expressed very early in the infection context, and the *agr* regulation was not implied at bacterial densities around $10^6$ CFU/mL (Bronner *et al*., 2003), contrary to *in vitro* conditions (Bronner *et al*., 2000). Proteomic investigations, rather than gene expression analysis, provided a better analysis of the effective and concerted production of virulence factors.

## APPLICATIONS AND ENGINEERED PORE-FORMING TOXINS IN PERSPECTIVE

Pore formation and the rupture in the ionic equilibrium of the cells led to the use of α-toxin to study cell response cascades or exocytosis (Anhert-Gilger *et al*., 1985; Bhakdi *et al*., 1993). Integration of the pore in an electrical circuit offers a model to study currents of low frequencies (30–120 Hz) (Misakian *et al*., 2002), according to the bulk pH and ionic strength (Kasianowicz *et al*., 2003). In fact, diverse small molecules (< 2000 Da) pass through the α-toxin pore, and the use of detectable tagged molecules with other analytes that can act as competitors allows quantification of these analytes (Kasianowicz *et al*., 2001).

Nucleic acid molecules (single-stranded DNA or RNA) traverse the α-toxin pore (Kasianowicz *et al*., 1996), and their microsecond time-scale transfer through the pore affects its electrical properties, so that event has been modeled with precision on experimental basis (Akeson *et al*., 1999; Hendrickson *et al*., 2000; Kasianowicz *et al*., 2001, 2002, 2004; Kong *et al*., 2002, 2004). Such studies are consistent with further applications using cell transfection and/or DNA-DNA recombination in isolated cells.

Because the β-stranded pore-forming toxins offer some structural robustness, modified toxins have been engineered and also tested in different fields. A modified α-toxin with an HisX5-tag produced a switch where pore activity can be (i) turned off by micromolar concentrations of divalent zinc ions and (ii) turned back on with the chelating agent EDTA (Walker *et al*., 1994). Such a switch was also shown to be operational to generate a limited number of pores for osmo-protection of cells with trehalose flowing in the pore, and it results in a significant yield of viable cells after thawing or anhydrobiosis (Russo *et al*., 1997; Eroglu *et al*., 2000; Chen *et al*., 2001). Trehalose, a non-toxic disaccharide, was used to enter some cell lines, and intracellular concentrations up to 0.5 M were reached and resulted in an appreciable yield of viable cells after thawing or anyhydrobiosis (Acker *et al*., 2003; Buchanan *et al*., 2004). $His_{5-}$ and $His_{4-}$ actuated switch-

able pores or mixtures of heteropores with the wild-type protein were also investigated for their sensing properties towards a series of divalent cations ($Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Cu^{2+}$) (Braha *et al*., 1997, 2000). The α-toxin pore has also been modified with 7 or 14 arginine residues pointing within the lumen, either of which can interact with β-cyclodextrin molecules, which in turn may block the pore (Cheley *et al*., 2002) or reverse the ionic selectivity (Gu *et al*., 2000). Exploration of biosensing has also been investigated toward the detection of organic molecules, such as olfactive or explosive compounds (Gu *et al*., 1999, 2001; Shin *et al*., 2002), and phosphate oxyanions, such as inositol triphosphate IP3 (Cheley *et al*., 2002). Specific tethered adapters can also be installed within the pore lumen so that the recognition of proteins (Movileanu *et al*., 2000; Howorka *et al*., 2004) or nucleic acid (Howorka *et al*., 2001a and b, 2002; Sanchez-Quesada *et al*., 2004) ligand blocks the pore function, giving again a specific electrical signature.

## CONCLUSION

The family of pore-forming toxins secreted by *Staphylococcus aureus* illustrates the incredible diversification of toxic compounds encountered in this bacteria. The different beta-barrel PFTs provide biological activity toward the wide variety of human blood cells, and several encoding genes can be genetically maintained in a single isolate, thus enhancing the leucotoxin combinations. This variety might constitute a benefit to the bacteria to escape the immune memory by the preservation of the biological activity of certain toxins at least. These toxins certainly constitute interesting tools for the bacteria to acquire the bulk of nutrients after cell lysis. The generation of cell ghosts constitute another pitfall for the cytotoxic immune response, physically and by the burst of inflammatory mediators contained in activated but lysed cells. With these qualities, beta-barrel PFTs represent interesting targets that can be targeted for the control infections. On this fundamental aspect, α-toxin and leukotoxins illustrate how complex the transition between a hydrosoluble state and the oligomeric and lipostabilized state is and, despite the fact that they operate similarly, several elements remain to be understood as the molecular targets of toxins, the association process of the constitutive proteins, and rules that define the robustness of oligomers. Nevertheless, their molecular architecture has proven the possibility of modification in order to control their ionic selectivity and their development as biosensors for a wide variety of molecules.

## ACKNOWLEDGMENTS

This review is dedicated to the memory of our regretted friend, and nice researcher, Gianfranco Menestrina (d. 2004).

## REFERENCES

Ahnert-Hilger, G., Bhakdi, S. and Gratzl, M. (1985). Minimal requirements for exocytosis: a study using PC12 cells permeabilized with staphylococcal alpha-toxin. *J. Biol. Chem*. **260,** 12730–12734.

Acker, J.P., Fowler, A., Lauman, B., Cheley, S. and Bayley, H. (2002). Survival of dessicacted mammalian cells: beneficial effects of isotonic media. *Cell Preserv. Technol*. **1,** 129–140.

Akeson, M., Branton, D., Kasianowicz, J.J., Brandin, E. and Deamer D.W. (1999). Microsecond time-scale discrimination among polycytidylic acid, polyadenylic acid, and polyuridylic acid as homopolymers or as segments within single RNA molecules. *Biophys J*. **77,** 3227–3233.

Baba Moussa, L., Sanni, A., Dagnra, A.Y., Anagonou, S., Prince-David, M., Edoh, V., Befort, J.J., Prévost, G. and Monteil, H. (1999a). Approche épidémiologique de l'antibiorésistance et de la production de leucotoxines par les souches de *Staphylococcus aureus* isolées en Afrique de l'Ouest. *Med. Mal. Infect*. **29,** 689–696.

Baba Moussa, L., Werner, S., Colin, D.A., Mourey, L., Pédelacq, J.D., Samama, J.P., Sanni, A., Monteil, H. and Prévost, G. (1999b). Discoupling the $Ca^{2+}$-activation from the pore-forming function of the bicomponent Panton-Valentine leucocidin in human PMNs. *FEBS Lett*. **461,** 280–286.

Baida, C., Budarina, Z.I., Kuzmin, N.P. and Solonin, A.S. (1999). Complete nucleotide sequence and molecular characterization of hemolysin II gene from *Bacillus cereus*. *FEMS Microbiol. Lett*. **180,** 7–14.

Bantel, H., Sinha, B., Domschke, W., Peters, G., Schulze-Osthoff, K. and Janicke, R.U. (2001). Alpha-toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J. Cell Biol*. **155,** 637–648.

Bashford, C. L., Alder, G. M., Fulford, L. G., Korchev, Y. E., Kovacs, E., MacKinnon, A., Pederzolli, C. and Pasternak, C. A. (1996). Pore formation by *S. aureus* alpha-toxin in liposomes and planar lipid bilayers: effects of nonelectrolytes. *J. Membr. Biol*. **150,** 37–45.

Belmonte, G., Cescatti, L., Ferrari, B., Nicolussi, T., Ropele, M. and Menestrina, G. (1987). Pore formation by *Staphylococcus aureus* alpha-toxin in lipid bilayers: dependence upon temperature and toxin concentration. *Eur. Biophys. J*. **14,** 349–358.

Bernheimer, A.W. (1974). Interactions between membranes and cytolytic bacterial toxins. *Biochim. Biophys. Acta* **344,** 27–50

Bezrukov, S.M. and Kasianowicz, J.J. (1993). Current noise reveals protonation kinetics and number of ionizable sites in an open protein ion channel. *Phys. Rev. Lett*. **70,** 2352–2355.

Bezrukov, S.M., Vodyanoy, I., Brutyan, R.A. and Kasianowicz, J.J. (1996). Dynamics and free energy of polymers partitioning into a nanoscale pore. *Macromolecules* **29,** 8517–8522.

Bhakdi, S and Tranum-Jensen, J. (1991). Alpha-toxin of *Staphylococcus aureus*. *Microbiol. Rev*. **55,** 733–751.

Bhakdi, S., Bayley, H., Valeva, A., Walev, I., Walker, B., Weller, U., Kehoe, M. and Palmer, M. (1996). Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins. *Arch. Microbiol*. **165,** 73–79.

Bhakdi, S., Füssle, R. and Tranum-Jensen, J. (1981) Staphylococcal alpha-toxin: oligomerization of hydrophilic monomers to form

amphiphilic hexamers induced through contact with deoxycholate detergent micelles. *Proc. Natl. Acad. Sci. USA* **78,** 5475–5479.

Bhakdi, S., Füssle, R., Utermann, G. and Tranum-Jensen, J. (1983). Binding and partial inactivation of *S. aureus* alpha-toxin by plasma low density lipoprotein. *J. Biol. Chem*. **258,** 5899–5904.

Bhakdi, S., Muhly, M., Korom, S. and Hugo, F. (1989) Release of interleukin-1β associated with potent cytocidal action of staphylococcal a-toxin on human monocytes. *Infect. Immun*. **57,** 3512–3519.

Bhakdi, S., Muhly, M., Mannhardt, U., Hugo, F., Klappetek, K., Mueller-Eckardt, C. and Roka, C. (1988). Staphylococcal alpha-toxin promotes blood coagulation via attack on human platelets. *J. Exp. Med*. **168,** 527–542.

Bhakdi, S., Weller, U., Walev, I., Martin, E., Jonas, D. and Palmer, M. (1993). A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microb. Immunol*. **182,** 167–175.

Braha, O., Walker, B., Cheley, S., Kasianowicz, J. J., Song, L., Gouaux, J. E. and Bayley, H. (1997). Designed protein pores as components for biosensors. *Chem. Biol*., **4,** 497–505.

Braha, O., Gu, L.Q., Zhou, L., Lu, X., Cheley, S. and Bayley, H. (2000). Simultaneous stochastic sensing of divalent metal ions. *Nat. Biotechnol*. **18,** 1005–1007.

Bramley, A.J., Patel, A.H., O'Reilly, M., Foster, R. and Foster, T.J. (1989). Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun*. **57,** 2489–2494.

Bronner, S., Jehl, F., Peter, J.D., Ploy, M.C., Renault, C., Arvis, P., Monteil, H. and Prévost, G. (2003). Moxifloxacin efficacy and vitreous penetration in a rabbit model of *Staphylococcus aureus* endophthalmitis and effect on gene expression of leucotoxins and virulence regulator factors. *Antimicrob. Agents Chemother*. **47,** 1621–1629.

Bronner, S., Stoessel, P., Gravet, A., Monteil, H. and Prévost, G. (2000). Variable expressions of *Staphylococcus aureus* bicomponent leucotoxins semiquantified by competitive reverse transcription-PCR. *Appl. Environ. Microbiol*. **66,** 3931–3938.

Browne, K.A., Blink, E., Sutton, V.R., Froelich, C.J., Jans, D.A. and Trapani, J.A. (1999). Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. *Mol. Cell Biol*. **19,** 8604–8615.

Buchanan, S.S., Gross, S.A., Acker, J.P., Toner, M., Carpenter, J.F. and Pyatt, D.W. (2004). Cryopreservation of stem cells using trehalose: evaluation of the method using a human hematopoietic cell line. *Stem Cells Dev*. **13,** 295–305.

Buerke, M., Sibelius, U., Grandel, U., Buerke, U., Grimminger, F., Seeger, W., Meyer, J. and Darius, H. (2002). *Staphylococcus aureus* alpha toxin mediates polymorphonuclear, leukocyte-induced vasocontraction and endothelial dysfunction. *Shock* **17,** 30–35.

Cescatti, L., Pederzolli, C. and Menestrina, G. (1991). Modification of lysine residues of *S. aureus* α-toxin: effects on its channel forming properties. *J. Membr. Biol*. **119,** 53–64.

Cheley, S., Malghani, M.S., Song, L., Hobaugh, M., Gouaux, J.E., Yang, J. and Bayley, H. (1997). Spontaneous oligomerization of a staphylococcal α-hemolysin conformationally constrained by removal of residues that form the transmembrane β-barrel. *Protein Eng*. **10,** 1433–1443.

Cheley, S., Gu, L.Q. and Bayley, H. (2002). Stochastic sensing of nanomolar inositol 1,4,5-trisphosphate with an engineered pore. *Chem. Biol*. **9,** 829–838.

Chen, T., Acker, J.P., Eroglu, A., Cheley, S., Bayley, H., Fowler, A. and Toner, M. (2001). Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells. *Cryobiology* **43,** 168–181.

Colin, D.A., Mazurier, I., Sire, S. and Finck-Barbançon, V. (1994). Interaction of the two components of leukocidin from Staphylococcus aureus with human polymorphonuclear leukocyte membranes: sequential binding and subsequent activation. *Infect. Immun*. **62,** 3184–3188.

Colin, D.A. and Monteil, H. (2003). Control of the oxidative burst of human neutrophils by staphylococcal leukotoxins. *Infect. Immun*. **71,** 3724–3729.

Comai, M., Dalla Serra, M., Coraiola, M., Werner, S., Colin, D.A., Monteil, H., Prévost, G. and Menestrina, G. (2002). Protein engineering modulates the transport properties and ion selectivity of the pores formed by staphylococcal gamma-hemolysins in lipid membranes. *Mol. Microbiol*. **44,** 1251–1267.

Cooney, J., Kienle, Z., Foster, T.J. and O'Toole, P.W. (1993). The gamma-hemolysin locus of *Staphylococcus aureus* comprises three linked genes, two of which are identical to the genes for the F and S components of leukocidin. *Infect. Immun*. **61,** 768–771.

Couppié, P., Cribier, B., Prévost, G., Grosshans, E. and Piémont, Y. (1994). Leucocidin from *Staphylococcus aureus* and cutaneous infections: an epidemiological study. *Arch. Dermatol*. **130,** 1208–1209.

Couppié, P., Hommel, D., Prévost, G., Godart, M.C., Moreau, B., Sainte-Marie, D., Peneau, C., Hulin, A., Monteil, H. and Pradinaud, R. (1997). Septicémie à *Staphylococcus aureus*, furoncle et leucocidine de Panton et Valentine: 3 observations. *Ann. Dermatol. Vénéréol*. **124,** 684–686.

Cribier, B., Prévost, G., Couppié, P., Finck-Barbançon, V., Grosshans, E. and Piémont, Y. (1992). *Staphylococcus aureus* leukocidin: a new virulence factor in cutaneous infections. *Dermatology* **185,** 175–180.

Czajkowsky, D.M., Sheng, S.T. and Shao, Z.F. (1998). Staphylococcal alpha-hemolysin can form hexamers in phospholipid bilayers. *J. Mol. Biol*. **276,** 325–330.

Dajcs, J.J., Austin, M.S., Sloop, G.D., Moreau, J.M., Hume, E.B., Thompson, H.W., McAleese, F.M., Foster, T.J. and O'Callaghan, R.J. (2002). Corneal pathogenesis of *Staphylococcus aureus* strain Newman. *Invest. Ophthalmol. Vis. Sci*. **43,** 1109–1115.

Dajcs, J.J., Thibodeaux, B.A., Girgis, D.O. and O'Callaghan, R.J. (2002). Corneal virulence of *Staphylococcus aureus* in an experimental model of keratitis. *DNA Cell Biol*. **21,** 375–382.

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and McKinnon, R. (1998). The structure of the potassium channel: molecular basis of $K^+$ conduction and selectivity. *Science* **280,** 69–77.

Dufourcq, J., Castano, S. and Talbot, J.C. (1999) δ-toxin, related hemolytic toxins and peptidic analogues. In: *Bacterial Protein Toxins: A Comprehensive Sourcebook* (eds Alouf J.E., Freer J.H) pp. 386–401. Academic Press, London.

Eroglu, A., Russo, M. J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H. and Toner, M. (2000). Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nat. Biotechnol*. **18,** 163–167.

Essmann, F., Bantel, H., Totzke, G., Engels, I.H., Sinha, B., Schulze-Osthoff K. and Janicke, R.U. (2003). *Staphylococcus aureus* alpha-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation. *Cell Death Differ*. **10,** 1260–1272.

Ferreras, M., Höper, F., Dalla Serra, M., Colin, D. A., Prévost, G. and Menestrina, G. (1998). The interaction of *Staphylococcus aureus* bicomponent gamma hemolysins and leucocidins with cells and model membranes. *Biochim. Biophys. Acta* **1414,** 108–126.

Finck-Barbançon, V., Duportail, G., Meunier, O. and Colin, D.A. (1993). Pore formation by a two-component leukocidin from *Staphylococcus aureus* within the membrane of human polymorphonuclear leukocytes. *Biochim. Biophys. Acta* **1182,** 275–282.

Fink, D., Contreras, M. L., Lelkes, P. I. and Lazarovici, P. (1989). *Staphylococcus* α-toxin activates phospholipases and induces a $Ca^{2+}$ influx in PC12 cells. *Cell. Signal*., **1,** 387–393.

Forti, S. and Menestrina, G. (1989). Staphylococcal alpha-toxin increases the permeability of lipid vesicles by a cholesterol and pH dependent assembly of oligomeric channels. *Eur. J. Biochem*. **181,** 767–773.

Freer, J. H., Arbuthnott, J. P. and Bernheimer, A. W. (1968). Interaction of staphylococcal alphatoxin with artificial and natural membranes. *J. Bacteriol*. **95,** 1153–1168.

Gauduchon, V., Werner, S., Prévost, G., Monteil, H. and Colin, D.A. (2001). Flow cytometric determination of Panton-Valentine leucocidin S component binding. *Infect. Immun*. **69,** 2390–2395.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J.C., Lina, G., Bes., M, Vandenesch, F., Piémont, Y., Brousse, N., Floret, D. and Etienne, J. (2002). Association between Staphylococcus aureus strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotizing pneumonia in young immunocompetent patients. *Lancet* **359,** 753–759.

Gouaux, E. (1997). Channel-forming toxins: tales of transformation. *Curr. Opin. Struct. Biol*. **7,** 566–573.

Gouaux, J.E., Braha, O., Hobaugh, M.R., Song, L., Cheley, S., Shustak, C. and Bayley, H. (1994). Subunit stoichiometry of staphylococcal α-hemolysin in crystals and on membranes: a heptameric transmembrane pore. *Proc Natl Acad Sci USA* **91,** 12828–12831.

Gravet, A. (2001). Characterization of the LukE-LukD leucotoxin from *Staphylococcus aureus* and *in vitro*, *in vivo* analysis of its expression. PhD Thesis, Institute of Bacteriology, Louis Pasteur University, Strasbourg, France.

Gravet, A., Colin, D.A., Keller, D., Girardot, R., Monteil, H. and Prévost, G. (1998). Characterization of a novel structural member, LukE-LukD, of the bicomponent leucotoxins family. *FEBS Lett*. **436,** 202–208.

Gravet, A., Couppié, P., Meunier, O., Clyti, E., Moreau, B., Pradinaud, R., Monteil, H. and Prévost, G. (2001). *Staphylococcus aureus* isolated from impetigo produces both epidermolysins A or B and LukE+LukD in 78% of 131 retrospective and prospective cases. *J. Clin. Microbiol*. **39,** 4349–4356.

Gravet, A., Rondeau, M., Harf-Monteil, C., Grunenberger, F., Monteil, H., Scheftel, J.M. and Prévost, G. (1999). Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-LukD. *J. Clin. Microbiol*. **37,** 4012–4019.

Gray, G.S. and Kehoe, M. (1984). Primary sequence of the alpha-toxin gene from *Staphylococcus aureus* Wood 46. *Infect. Immun*. **46,** 615–618.

Gu, L.Q., Braha, O., Conlan, S., Cheley, S. and Bayley, H. (1999). Stochastic sensing of organic analytes by a pore-forming protein containing a molecular adapter. *Nature* **398,** 686–690.

Gu, L.Q., Dalla Serra, M., Vincent, J.B., Vigh, G., Cheley, S., Braha, O. and Bayley, H. (2000). Reversal of charge selectivity in transmembrane protein pores by using noncovalent molecular adapters. *Proc. Natl. Acad. Sci. USA*. **97,** 3959–3964.

Gu, L.Q., Cheley, S. and Bayley, H. (2001). Capture of a single molecule in a nanocavity. *Science*. **291,** 636–640.

Guillet, V., Keller, D., Prévost, G. and Mourey, L. (2004). Crystallization and preliminary crystallographic data of a leucotoxin S component from *Staphylococcus aureus*. *Acta Crystallogr. D Biol. Crystallogr*. **60,** 310–313.

Guillet, V., Roblin, P., Werner, S., Coraiola, M., Menestrina, G., Monteil, H., Prévost, G. and Mourey, L. (2004). Crystal structure of leucotoxin S component: new insight into the staphylococcal beta-barrel pore-forming toxins. *J. Biol. Chem*. **279,** 41028–41037.

Guyonnet, F. and Plommet, M. (1970). Hémolysine gamma de *Staphylococcus aureus*: purification et propriétés. *Ann. Inst. Pasteur* **118,** 19–33.

Haslinger, B., Strangfeld, K., Peters, G., Schulze-Osthoff, K. and Sinha, B. (2003). *Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumor necrosis factor-alpha and the mitochondrial death pathway. *Cell Microbiol*. **5,** 729–741.

Hayashi, T., Kamio, Y., Hishinuma, F., Usami, Y., Titani, K. and Terawaki, Y. (1989). *Pseudomonas aeruginosa* cytotoxin: the nucleotide sequence of the gene and the mechanism of activation of the protoxin. *Mol. Microbiol*. **3,** 861–868.

Hensler, T., Köller, M., Prévost, G., Piémont, Y. and König, W. (1994a). GTP-binding proteins are involved in the modulated activity of human neutrophils treated by the Panton-Valentine leucocidin from *Staphylococcus aureus*. *Infect. Immun*. **62,** 5281–5289.

Hensler, T., König, B., Prévost, G., Piémont, Y., Köller, M. and König, W. (1994b). LTB4- and DNA fragmentation induced by leukocidin from *Staphylococcus aureus*. The protective role of GM-CSF and G-CSF on human neutrophils. *Infect. Immun*. **62,** 2529–2535.

Henrickson, S.E., Misakian, M., Robertson, B. and Kasianowicz, J.J. (2000). Driven DNA transport into an asymmetric nanometer-scale pore. *Phys. Rev. Lett*. **85,** 3057–3060.

Howorka, S., Cheley, S. and Bayley, H. (2001a). Sequence-specific detection of individual DNA strands using engineered nanopores. *Nat. Biotechnol*. **19,** 636–639.

Howorka, S., Movileanu, L., Braha, O. and Bayley, H. (2001). Kinetics of duplex formation for individual DNA strands within a single protein nanopore. *Proc. Natl. Acad. Sci. USA*. **98,** 12996–13001.

Howorka, S. and Bayley, H. (2002). Probing distance and electrical potential within a protein pore with tethered DNA. *Biophys. J*. **83,** 3202–3210.

Howorka, S., Nam, J., Bayley, H. and Kahne, D. (2004). Stochastic detection of monovalent and bivalent protein-ligand interactions. *Angew Chem Int Ed Engl*. **43,** 842–846.

Hume, E.B., Dajcs, J.J., Moreau, J.M. and O'Callaghan, R.J. (2000). Immunization with alpha-toxin toxoid protects the cornea against tissue damage during experimental *Staphylococcus aureus* keratitis. *Infect. Immun*. **68,** 6052–6055.

Hunter, S.E., Brown, J.E., Oyston, P.C.F., Sakurai, J. and Titball, R.W. (1993). Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, gamma-hemolysin, and leukocidin of *Staphylococcus aureus*. *Infect. Immun*. **61,** 3958–3965.

Jonas, D., Walev, I., Berger, T., Liebetrau, M., Palmer, M. and Bhakdi, S. (1994). Novel path to apoptosis: small transmembrane pores created by staphylococcal α-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect. Immun*. **62,** 1304–1312.

Jursch, R., Hildebrand, A., Hobom, G., Tranum-Jensen, J., Ward, R., Kehoe, M. and Bhakdi, S. (1994). Histidine residues near the N-terminus of *Staphylococcus* alpha-toxin as reporters of regions that are critical for oligomerization of pore formation. *Infect. Immun*. **62,** 2249–2256.

Kamio, Y., Rahman, A., Nariya, H., Ozawa, T. and Izaki, K. (1993). The two staphyloccocal bicomponent toxins, leukocidin and gamma-hemolysin, share one component in common. *FEBS Lett*. **321,** 15–18.

Kaneko, J., Kimura, T., Narita, S., Tomita, T. and Kamio, Y. (1998). Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. *Gene* **17,** 215, 57–67.

Kaneko, J., Muramoto, K. and Kamio, Y. (1997). Gene of LukF-PV-like component of Panton-Valentine leukocidin in *Staphylococcus aureus* P83 is linked with *luk*M. *Biosc. Biotech. Biochem*. **61,** 541–544.

Kasianowicz, J. J. and Bezrukov, S. M. (1995). Protonation dynamics of the alpha-toxin ion channel from spectral analysis of pH-dependent current fluctuations. *Biophys. J.* **69,** 94–105.

Kasianowicz, J. J., Brandin, E., Branton, D. and Deamer, D. W. (1996). Characterization of individual polynucleotide molecules using a membrane channel. *Proc. Natl. Acad. Sci. USA* **93,** 13770–13773.

Kasianowicz, J.J. and Bezrukov, S.M. (1995). Protonation dynamics of the alpha-toxin ion channel from spectral analysis of pH-dependent current fluctuations. *Biophys. J.* **69,** 94–105.

Kasianowicz, J.J., Henrickson, S.E., Weetall, H.H. and Robertson, B. (2001). Simultaneous multianalyte detection with a nanometer-scale pore. *Anal. Chem*. **73,** 2268–2272.

Kasianowicz, J.J. (2002). Nanometer-scale pores: potential applications for analyte detection and DNA characterization. *Dis. Markers*. **18,** 185–191.

Kasianowicz, J.J. (2004). Nanopores: flossing with DNA. *Nat. Mater*. **3,** 355–356.

Katsumi, H., Tomita, T., Kaneko, J. and Kamio, Y. (1999). Vitronectin and its fragments purified as serum inhibitors of *Staphylococcus aureus* γ-hemolysin and leucocidin, and their specific binding to the Hlg2 and the LukS components of the toxins. *FEBS Lett*. **460,** 451–456.

Kawate, T. and Gouaux, E. (2003) Arresting and releasing Staphylococcal alpha-hemolysin at intermediate stages of pore formation by engineered disulfide bonds. *Protein Sci*. **12,** 997–1006.

Köller, M., Hensler, T., König, B., Prévost, G., Alouf, J. and König, W. (1993). Induction of heatshock proteins by bacterial toxins, lipid mediators, and cytokines in human leucocytes. *Zentralbl. Bakteriol*. **278,** 365–376.

Kong, C.Y. and Muthukumar, M. (2002). Modeling of polynucleotide translocation through protein pores and nanotubes. *Electrophoresis* **26,** 2697–2703.

Kong, C.Y. and Muthukumar, M. (2004). Polymer translocation through a nanopore.II. Excluded volume effect. *J. Chem. Physics* **120,** 3460–3466.

König, B., Köller, M., Prévost, G., Piémont, Y., Alouf, J.E., Schreiner, A. and König, W. (1994). Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, erythrogenic toxin A): generation of interleukin-8. *Infect. Immun*. **62**:4831–4837.

König, B., Prévost, G. and König, W. (1997). Composition of staphylococcal bicomponent toxins determines pathophysiological reactions. *J. Med. Microbiol*. **46,** 479–485.

König, B., Prévost, G., Piémont, Y. and König, W. (1995). Effects of *Staphylococcus aureus* leucocidins inflammatory mediator release from human granulocytes. *J. Infect. Dis*. **171,** 607–613.

Korchev, Y. E., Alder, G. M., Bakhramov, A., Bashford, C. L., Joomun, B. S., Sviderskaya, E. V., Usherwood, P. N. R. and Pasternak C. A. (1995a). *Staphylococcus aureus* alpha-toxin-induced pores: channel-like behavior in lipid bilayers and clamped cells. *J. Membr. Biol*. **143,** 143–151.

Korchev, Y. E., Bashford, C. L., Alder, G. M., Kasianowicz, J. J. and Pasternak, C. A. (1995b). Low conductance states of a single ion channel are not "closed." *J. Membr. Biol*. **147,** 233–239.

Krasilnikov, O.V., Sabirov, R.Z., Ternovsky, O.V., Merzlyak, P.G. and Muratkhodjaev, J.N. (1992). A simple method for the determination of the pore radius of channels in planar lipid bilayer membranes. *FEMS Microbiol. Immunol*. **105,** 93–100.

Krasilnikov, O.V., Ternovskii, V.I., Sabirov, R.Z., Zaripova, R.K. and Tashmukhamedov, B.A. (1986). Cationic-anionic selectivity of staphylotoxin channels in lipid bilayer. *Biophysics* **31,** 658–663.

Krishnasastry, M., Walker, B., Braha, O. and Bayley, H. (1994). Surface labeling of key residues during assembly of the transmembrane pore formed by staphylococcal α-hemolysin. *FEBS Lett*. **356,** 66–71.

Kuroda, M., Ohta, T., Ushiyama, I., Baba, T., Yuzawa, H., Kobayashi, I. *et al*. (2001). Whole genome sequencing of methicillin-resistant *Staphylococcus aureus. Lancet*, **357,** 1225–1239.

Lala, A. and Raja, S.M. (1995). Photolabeling of a pore-forming toxin with the hydrophobic probe 2-[$^3$H]diazofluorene. *J. Biol. Chem*. **270,** 11348–11357.

Liassine, N., Auckenthaler, R., Descombes, M.C., Bes, M., Vandenesch, F. and Etienne, J. (2004). Community-acquired methicillin-resistant *Staphylococcus aureus* isolated in Switzerland contains the Panton-Valentine leukocidin or exfoliative toxin genes. *J. Clin. Microbiol*. **42,** 825–828.

Lina, G., Piémont, Y., Godail-Gamot, F., Bès, M., Peter, M.O., Vandenesch, F. and Jérôme, E. (1999). Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin. Infect. Dis*. **29,** 1128–1132.

Lund, T., De Buyser, M.L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. **29,** 254–261.

Malghani, M.S., Fang, Y., Cheley, S., Bayley, H. and Yang J. (1999). Heptameric structures of two alpha-hemolysin mutants imaged with *in situ* atomic force microscopy. *Microsc. Res. Tech*. **44,** 353–356.

McElroy, M.C., Harty, H.R., Hosford, G.E., Boylan, G.M., Pittet, J.F. and Foster, T.J. (1999). Alpha-toxin damages the air-blood barrier of the lung in a rat model of *Staphylococcus aureus*-induced pneumonia. *Infect. Immun*. **67,** 5541–5544.

Melton, J.A., Parker, M.W., Rossjohn, J., Buckley, J.T. and Tweten, R.K. (2004). The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. *J. Biol. Chem*. **279,** 14315–14322.

Menestrina, G. (1986). Ionic channels formed by *Staphylococcus aureus* alpha-toxin: voltage dependent inhibition by di- and trivalent cations. *J. Membr. Biol*. **90,** 177–190.

Menestrina, G. and Vécsey-Semjén, B. (1999). Biophysical methods and model membranes for the study of bacterial pore-forming toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds J. E. Alouf and J. H. Freer). Academic Press, London. pp. 287–309.

Menestrina, G., Dalla Serra, M., Comai, M., Coraiola, M., Viero, G., Werner, S., Colin, D.A., Monteil, H. and Prévost, G. (2003). Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus. FEBS Lett*. **552,** 54–60.

Menestrina, G., DallaSerra, M.D. and Prévost, G. (2001). Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* **39,** 1661–1672.

Menzies, B.E. and Kernodle, D.S. (1994). Site-directed mutagenesis of the alpha-toxin gene of *Staphylococcus aureus*: role of histidines in toxin activity *in vitro* and in a murine model. *Infect. Immun*. **62,** 1843–1847.

Menzies, B.E. and Kourteva I. (2000). *Staphylococcus aureus* alpha-toxin induces apoptosis in endothelial cells. *FEMS Immunol. Med. Microbiol*. **29,** 39–45.

Meunier, O., Falkenrodt, A., Monteil, H. and Colin, D. A. (1995). Application of flow cytometry in toxinology: pathophysiology of human polymorphonuclear leucocytes damaged by a pore-forming toxin from *Staphylococcus aureus. Cytometry* **21,** 241–247.

Meunier, O., Ferreras, M., Supersac, G., Hoeper, F., Baba Moussa, L., Monteil, H., Colin, D.A., Menestrina, G. and Prévost, G. (1997). A predicted α-sheet from class S components of staphylococcal α-hemolysin is essential for the secondary interaction of the class F component. *Biochim. Biophys. Acta* **1326,** 275–289.

Miles, G., Cheley, S., Braha, O. and Bayley, H. (2001). The staphylococcal leukocidin bicomponent toxin forms large ionic channels. *Biochemistry*. **40,** 8514–8522.

Miles, G., Movileanu, L. and Bayley, H. (2002). Subunit composition of a bicomponent toxin: staphylococcal leukocidin forms an octameric transmembrane pore. *Protein Sci*. **11,** 894–902.

Misakian, M. and Kasianowicz, J.J. (2003). Electrostatic influence on ion transport through the alphaHL channel. *J. Membr. Biol*. **195,** 137–146.

Moreau, J.M., Sloop, G.D., Engel, L.S., Hill, J.M. and O'Callaghan, R.J. (1997). Histopathological studies of staphylococcal alpha-toxin: effects on rabbit corneas. *Curr. Eye Res*. **16,** 1221–1228.

Morinaga, N., Kaihou, Y. and Noda, M. (2003). Purification, cloning, and characterization of variant LukE-LukD with strong leukocidal activity of staphylococcal bicomponent leukotoxin family. *Microbiol. Immunol*. **47,** 81–90.

Movileanu, L., Howorka, S., Braha, O. and Bayley, H. (2000). Detecting protein analytes that modulate transmembrane movement of a polymer chain within a single protein pore. *Nat. Biotechnol*. **18,** 1091–1095.

Narita, S., Kaneko, J., Chiba, J. I., Piémont, Y., Jarraud, S., Etienne, J. and Kamio, Y. (2001). Phage conversion of Panton–Valentine leucocidin in *Staphylococcus aureus*: molecular analysis of a PVLconverting phage, SLT. *Gene* **268,** 195–206.

Nguyen, V.T., Higuchi, H. and Kamio, Y. (2002). Controlling pore assembly of staphylococcal gamma-hemolysin by low temperature and by disulphide bond formation in double-cysteine LukF mutants. *Mol. Microbiol*. **45,** 1485–1498.

Nguyen, V.T., Kamio, Y. and Higuchi, H. (2003). Single-molecule imaging of cooperative assembly of gamma-hemolysin on erythrocyte membranes. *EMBO J.* **22,** 4968–4979.

Nilsson, I.M., Hartford, O., Foster, T. and Tarkowski, A. (1999). Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. *Infect. Immun*. **67,** 1045–1049.

O'Callaghan, R.J., Callegan, M.C., Moreau, J.M., Green, L.C., Foster, T.J., Hartford, O.M., Engel, L.S. and Hill, J.M. (1997). Specific roles of alpha-toxin and beta-toxin during *Staphylococcus aureus* corneal infection. *Infect. Immun*. **65,** 1571–1578.

O'Callaghan, R.J., Girgis, D.O., Dajcs, J.J. and Sloop, G.D. (2003). Host defense against bacterial keratitis. *Ocul. Immunol. Inflamm*. **11,** 171–181.

Olson, R., Nariya, H., Yokota, K., Kamio, Y. and Gouaux, E. (1999). Crystal structure of staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nat. Struct. Biol*. **6,** 134–140.

O'Reilly, M., deAzavedo, J.C.S., Kennedy, S. and Foster, T.J. (1986). Inactivation of the alpha-hemolysin gene of *Staphylococcus aureus* 8325–4 by site-directed mutagenesis and studies of the expression of its hemolysis. *Microb. Pathogen*. **1,** 125–131.

Panchal, R.G. and Bayley, H. (1995). Interactions between residues in staphylococcal alpha-hemolysin revealed by reversion mutagenesis. *J. Biol. Chem*. **270,** 23072–23076.

Panton, P.N. and Valentine, F.C.O. (1932). Staphylococcal toxin. *Lancet* **222,** 506–508.

Pany, S., Vijayvargia, R. and Krishnasastry, M.V. (2004). Caveolin-1 binding motif of alpha-hemolysin: its role in stability and pore formation. *Biochem. Biophys. Res. Commun*. **322,** 29–36.

Parker, M.W., Buckley, J.T., Postma, J.P., Tucker, A.D., Leonard, K., Pattus, F. and Tsernoglou, D. (1994). Structure of the Aeromonas toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367,** 292–295.

Patel, A., Nowlan, H.P., Weavers, E.D. and Foster, T.J. (1987). Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infect. Immun*. **55,** 3103–3110.

Paula, S., Akeson, M. and Deamer, D. (1999). Water transport by the bacterial channel α-hemolysin. *Biochim. Biophys. Acta* **1418,** 117–126.

Pédelacq, J.D., Maveyraud, L., Prévost, G., Baba-Moussa, L., González, A., Courcelle, E., Shepard, W., Monteil, H., Samama, J.P. and Mourey, L. (1999). The structure of a *Staphylococcus aureus* leucocidin component (LukF-PV) reveals the fold of the water-soluble species of a family of transmembrane pore-forming toxins. *Structure* **7,** 277–287.

Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J. and Schlievert, P. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J. Bacteriol*. **170,** 4365–4372.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Pokorny, A., Birkbeck, T.H. and Almeida, P.F. (2002). Mechanism and kinetics of delta-lysin interaction with phospholipid vesicles. *Biochemistry* **41,** 11044–11056.

Prévost, G. (1999). The bicomponent staphylococcal leucotoxins and γ-hemolysins (toxins). In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds J. E. Alouf and J. H. Freer). Academic Press, London. pp. 402–418.

Prévost, G. (2005). Toxins in *Staphylococcus aureus* pathogenesis. In: *Microbial Toxins: Molecular and Cellular Biology* (ed. T. Proft) pp. 243–283, Horizon Bioscience Press, Norfolk.

Prévost, G., Bouakham, T., Piémont, Y. and Monteil, H. (1995a). Characterization of a synergohymenotropic toxin from *Staphylococcus intermedius*. *FEBS Lett*. **376,** 135–140.

Prévost, G., Couppié, P., Prévost, P., Gayet, S., Petiau, P., Cribier, B., Monteil, H. and Piémont, Y. (1995b). Epidemiological data on *Staphylococcus aureus* strains producing synergohymenotropic toxins. *J. Med. Microbiol*. **42,** 237–245.

Prévost, G., Cribier, B., Couppié, P., Petiau, P., Supersac, G., Finck-Barbançon, V., Monteil, H. and Piémont, Y. (1995c). Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immun*. **63,** 4121–4129.

Prévost, G., Colin, D.A., Mourey, L. and Menestrina, G. (2001). Staphylococcal pore-forming toxins. In: *Pore-forming Toxins* (ed. F. G. van der Goot). *Current Top. Microbiol. Immunol*. **257,** 53–83.

Prévost, G., Menestrina, G., Colin, D.A., Werner, S., Bronner, S., Dalla Serra, M., Baba Moussa, L., Coraiola, M., Gravet, A. and Monteil, H. (2003). Staphylococcal bicomponent leucotoxins, mechanism of action, impact on cells and contribution to virulence. In: *Pore-forming Peptides and Protein Toxins* (eds. G. Menestrina, M. Dalla Serra, P. Lazarovici) pp. 3–26. Taylor & Francis Press, London.

Pruefer, D., Makowski, J., Schnell, M., Buerke, U., Dahm, M., Oelert, H., Sibelius, U., Grandel, U., Grimminger, F., Seeger, W., *et al*. (2002). Simvastatin inhibits inflammatory properties of *Staphylococcus aureus* alpha-toxin. *Circulation* **106,** 2104–2110.

Rainard, P., Corrales, J.C., Barrio, M.B., Cochard, T. and Poutrel, B. (2003). Leucotoxic activities of Staphylococcus aureus strains isolated from cows, ewes, and goats with mastitis: importance of LukM/LukF'-PV leukotoxin. *Clin. Diagn. Lab. Immunol*. **10,** 272–277.

Rose, F., Dahlem, G., Guthmann, B., Grimminger, F., Maus, U., Hanze, J., Duemmer, N., Grandel, U., Seeger, W. and Ghofrani, H.A. (2002). Mediator generation and signaling events in alveolar epithelial cells attacked by *S. aureus* alpha-toxin. *Am. J. Physiol. Lung Cell. Mol. Physiol*. **282,** L207–214.

Russo, M.J., Bayley, H. and Toner, M. (1997). Reversible permeabilization of plasma membranes with an engineered switchable pore. *Nat. Biotechnol*. **15,** 278–282.

Sakoulas, G., Eliopoulos, G.M., Moellering, R.C., Wennersten, C., Venkataraman, L., Novick, R.P. and Gold, H.S. (2002). Accessory gene regulator (*agr*) locus in geographically diverse *Staphylococcus*

*aureus* isolates with reduced susceptibility to vancomycin. *Antimicrob. Agents Chemother*. **46,** 1492–1502.

Sanchez-Quesada, J., Saghatelian, A., Cheley, S., Bayley, H. and Ghadiri, M.R. (2004). Single DNA rotaxanes of a transmembrane pore protein. *Angew. Chem. Int. Ed. Engl*. **43,** 3063–3067.

Shin, S.H., Luchian, T., Cheley, S., Braha, O. and Bayley, H. (2002). Kinetics of a reversible covalent bond-forming reaction observed at the single molecule level. *Angew. Chem. Int. Ed. Engl*. **41,** 3707–3709.

Schwan, W.R., Langborne, M.H., Ritchie, H.D. and Stover, C.K. (2003). Loss of hemolysin expression in *Staphylococcus aureus agr* mutants correlates with selective survival during mixed infections in murine abscesses and wounds. *FEMS Immunol. Med. Microbiol*. **38,** 23–28.

Seeger, W., Birkenmeyer, R.G., Ermert, N., Suttorp, N., Bhakdi, S. and Dunker, H.R. (1990). Staphylococcal alpha-toxin-induced vascular leakage in isolated perfused rabbit lungs. *Lab. Investig*. **63,** 341–349.

Sibelius, U., Grandel, U., Buerke, M., Mueller, D., Kiss, L., Kraemer, H.J., Braun-Dullaeus, R., Haberbosch, W., Seeger, W. and Grimminger, F. (2000). Staphylococcal alpha-toxin provokes coronary vasoconstriction and loss in myocardial contractility in perfused rat hearts: role of thromboxane generation. *Circulation* **101,** 78–85.

Siqueira, J.A., Speeg-Schatz, C., Freitas, F.I.S., Sahel, J., Monteil, H. and Prévost, G. (1997). Channel-forming leucotoxins from *Staphylococcus aureus* cause severe inflammatory reactions in a rabbit eye model. *J. Med. Microbiol*. **46,** 486–494.

Smith, M.L. and Price, S.A. (1938). *Staphylococcus* γ-hemolysin. *J. Pathol. Bacteriol*. **47,** 379–393.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Staali, L., Monteil, H. and Colin, D. A. (1998). The pore-forming leukotoxins from *Staphylococcus aureus* open $Ca^{2+}$ channels in human polymorphonuclear neutrophils. *J. Membr. Biol*. **162,** 209–216.

Sugawara, N., Tomita, T. and Kamio, Y. (1997). Assembly of *Staphylococcus aureus* gamma-hemolysin into a pore-forming ring-shaped complex on the surface of human erythrocytes. *FEBS Lett*. **410,** 333–337.

Sugawara-Tomita, N., Tomita, T. and Kamio, Y. (2002). Stochastic assembly of two-component staphylococcal gamma-hemolysin into heteroheptameric transmembrane pores with alternate subunit arrangements in ratios of 3:4 and 4:3. *J. Bacteriol*. **184,** 4747–4756.

Supersac, G., Piémont, Y., Kubina, M., Prévost, G. and Foster, T. J. (1998). Assessment of the role of gamma-toxin in experimental endophthalmitis using an *hlg* deficient mutant of *Staphylococcus aureus*. *Microb. Pathog*. **24,** 241–251.

Suttorp, N., Fuhrmann, M., Tannert-Otto, S., Grimminger, F. and Bhadki, S. (1993). Pore-forming bacterial toxins potently induce release of nitric oxide in porcine endothelial cells. *J. Exp. Med*. **178,** 337–341.

Suttorp, N., Seeger, W., Dewein, E., Bhakdi, S. and Roka, L. (1985). Staphylococcal alpha-toxin-induced PGI2 production in endothelial cells: role of calcium. *Am. J. Physiol*. **248,** C127–134.

Suttorp, N., Seeger, W., Zucker-Reimann, J., Roka, L. and Bhakdi, S. (1987). Mechanism of leukotriene generation in polymorphonuclear leukocytes by staphylococcal alpha-toxin. *Infect. Immun*. **55,** 104–110.

Szmigielski, S., Sobiczewska, E., Prévost, G., Monteil, H., Colin, D. A. and Jeljaszewicz, J. (1998). Effects of purified staphylococcal leukocidal toxins on isolated blood polymorphonuclear leukocytes and peritoneal macrophages *in vitro*. *Zentralbl. Bakteriol*. **288,** 383–394.

Tappin, M.J., Pastore, A., Norton, R.S., Freer, J.H. and Campbell, I.D. (1988). High-resolution H-NMR study of the solution structure of resolution α-hemolysin. *Biochemistry* **27,** 1643–1647.

Thibodeau, A., Yao, X. and Forte, J.G. (1994). Acid secretion in α-toxin-permeabilized gastric glands. *Biochem. Cell Biol*. **72,** 26–35.

Valeva, A., Weisser, A., Walker, B., Kehoe, M., Bhakdi, S. and Palmer, M. (1996). Molecular architecture of toxin pore: a 15-residue sequence lines the transmembrane channel of staphylococcal α-toxin. *EMBO J*. **15,** 1857–1864.

Valeva, A., Palmer, M. and Bhakdi, S. (1997a). Staphylococcal alpha-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. *Biochemistry* **36,** 13298–13304.

Valeva, A., Pongs, J., Bhakdi, S. and Palmer, M. (1997b). Staphylococcal alpha-toxin: the role of the N-terminus in formation of the heptameric pore—a fluorescence study. *Biochim. Biophys. Acta*. **1325,** 281–286.

Valeva, A., Walev, I., Pinkernell, M., Walker, B., Bayley, H., Palmer, M. and Bhakdi, S. (1997c). Transmembrane beta-barrel of staphylococcal alpha-toxin forms in sensitive but not in resistant cells. *Proc Natl Acad Sci USA*. **94,** 11607–11611.

Valeva, A., Walev, I., Gerber, A., Klein, J., Palmer, M. and Bhakdi, S. (2000). Staphylococcal alphatoxin: repair of a calcium-impermeable pore in the target cell membrane. *Mol. Microbiol*. **36,** 467–476.

Valeva, A., Schnabel, R., Walev, I., Boukhallouk, F., Bhakdi, S. and Palmer, M. (2001). Membrane insertion of the heptameric staphylococcal alpha-toxin pore. A domino-like structural transition that is allosterically modulated by the target cell membrane. *J. Biol. Chem*. **276,** 14835–14841.

Vandenesch, F., Naimi, T., Enright, M,C., Lina, G., Nimmo, G.R., Heffernan, H., Liassine, N., Bes, M., Greenland, T., Reverdy, M.E. and Etienne, J. (2003). Community-acquired, methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg. Infect. Dis*. **9,** 978–984.

van der Flier, M., van Dijk, N.B., Fluit, A.C., Fleer, A., Wolfs, T.F. and van Gestel, J.P. (2003). Fatal pneumonia in an adolescent due to community-acquired, methicillin-resistant *Staphylococcus aureus* positive for Panton-Valentine-leukocidin. *Ned. Tijdschr. Geneeskd*. **147,** 1076–1079.

Vécsey-Semjén, B., Lesieur, C., Möllby, R. and van der Goot, F.G. (1997). Conformational changes due to membrane binding and channel formation by staphylococcal α-toxin. *J. Biol. Chem*. **272,** 5709–5717.

von Eiff, C., Heilmann, C., Proctor, R.A., Woltz, C., Peters, G. and Gotz, F. (1997). A site-directed *Staphylococcus aureus hem hem*B mutant is a small-colony variant which persists intracellularly. *J. Bacteriol*. **179,** 4706–4712.

Walev, I., Martin, E., Jonas, D., Mohamadzadeh, M., Müller-Klieser, W., Kunz, L. and Bhakdi, S. (1993). Staphylococcal alpha-toxin kills human keratinocytes by permeabilizing the plasma membrane for monovalent ions. *Infect. Immun*. **61,** 4972–4979.

Walev, I., Palmer, M., Martin, M., Jonas, D., Weller, U., Höhn-Bentz, H., Husmann, M. and Bhakdi, S. (1994). Recovery of human fibroblasts from attack by the pore-forming α-toxin of *Staphylococcus aureus*. *Microb. Pathogenesis* **17,** 187–201.

Walker, B., Braha, O., Cheley, S. and Bayley, H. (1995). An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch. *Chem. Biol*. **22,** 99–105.

Wang, X., Noda, M. and Kato, I. (1990). Stimulatory effect of staphylococcal leucocidin on phosphoinositide metabolism in rabbit polymorphonuclear leucocytes. *Infect. Immun*., **58,** 2745–2749.

Ward, P. D. and Turner, W. H. (1980). Identification of staphylococcal Panton–Valentine leukocidin as a potent dermonecrotic toxin. *Infect. Immun*. **28,** 393–397.

Ward, R.J., Palmer, M., Leonard, K. and Bhakdi, S. (1994). Identification of a putative membrane-inserted segment in the alpha-toxin of *Staphylococcus aureus*. *Biochemistry* **33,** 7477–7484.

Warny, M. and Kelly, C.P. (1999). Monocytic cell necrosis is mediated by potassium depletion and caspase-like proteases. *Am. J. Physiol*. **276,** C717–724.

Watanabe, M., Tomita, T. and Yasuda, T. (1987). Membrane-damaging action of staphylococcal alpha-toxin on phospholipid cholesterol liposomes. *Biochim. Biophys. Acta* **898,** 257–265.

Werner, S. (2001) Structural and functional aspects of the interactions of leucotoxins from *Staphylococcus aureus* with human leucocytes. PhD Thesis, Institut of Bacteriology, Louis Pasteur University, Strasbourg, France.

Werner, S., Colin, D.A., Coraiola, M., Menestrina, G., Monteil, H. and Prévost, G. (2002). Retrieving biological activity from LukF-PV mutants combined with different S components implies compatibility between the stem domains of these staphylococcal bicomponent leucotoxins. *Infect. Immun*. **70,** 1310–1318.

Woodin, A.M. (1960). Purification of the two components of leukocidin from *Staphylococcus aureus*. *Biochem. J*. **75,** 158–165.

Zou, D., Kaneko, J., Narita, S. and Kamio, Y. (2000). Prophage, phiPV83-pro, carrying panton-valentine leukocidin genes on the *Staphylococcus aureus* P83 chromosome: comparative analysis of the genome structures of phiPV83-pro, phiPVL, phi11, and other phages. *Biosci. Biotechnol. Biochem*. **64,** 2631–2643.

CHAPTER

# 33

# Aerolysin and related *Aeromonas* toxins

*Laure Gurcel, Ioan Iacovache, and F. Gisou van der Goot*

## INTRODUCTION

As contributors to their virulence, many pathogenic bacteria produce protein toxins. The largest class of these is composed by the pore-forming toxins (PFTs), which make up some 25% of all toxins. Some bacteria produce PFTs directed towards other bacteria, such as colicin producing *E. coli*. Most bacteria, however, produce PFTs directed towards eucaryotic host cells. These latter toxins share a similar mode of action despite their lack of sequence homology. The toxin is produced in a water-soluble form that may or not require proteolytic processing. The toxin then diffuses towards the target cell with which it binds with high affinity via the interaction with a receptor, which can either be a protein, a lipid, or a carbohydrate moiety. Cell surface concentration of the toxin promotes oligomerization into a ring-like structure that subsequently inserts into the membrane and forms a pore. Production of PFTs is common to Gram-positive and Gram-negative bacteria and is a phenomenon also found in parasites.

One member of this large family of PFTs is aerolysin produced by *Aeromonas* species. *Aeromonas* species are Gram-negative bacteria found in fresh water environments throughout the world (Austin *et al*., 1996). Members of the genus are pathogenic to many species, including amphibians, fish, reptiles, and humans and are a major source of economic losses in aquaculture (Altwegg and Geiss, 1989). In humans, *Aeromonas* spp., including *A. hydrophila, A. caviae, A. veronii biovar sobria, A. sobria, A. jandaei*, and *A. veronii biovar veronii,* have been involved in wound infections, sepsis, outbreaks of water- and food-borne gastroenteritis (Kirov, 1997; Janda and Abbott, 1998), and is an emerging pathogen in immuno-compromised hosts. Analysis of poultry, fish, and shrimp has indicated that more than 50% of commercial raw food samples and 20–30% of processed and ready-to-eat food samples contained *Aeromonas* species (Kingombe *et al*., 1999). In addition to commercial food, a recent study showed that fish caught off the Indian coast were frequently (37% of the isolates) contaminated with *A. hydrophila*, which were not only hemolytic but also multiple resistant to antibiotics (Thayumanavan *et al*., 2003).

Among the variety of potential virulence factors produced by *Aeromonas* spp., aerolysin has been considered the most important toxin, as illustrated by the facts that aerolysin is lethal to mice (Chopra *et al*., 2000), that immunization against the toxin leads to protection towards the bacterium, and that aerolysin-deficient strains are significantly less toxic than the parental strain in a mouse model (Chakraborty *et al*., 1987).

There is an ongoing debate regarding the nomenclature of this toxin. Very similar aerolysin-like molecules have been named: small beta, Greek-hemolysin, cytolytic enterotoxin, Act, and aerolysin. However, given their similarities in sequence (Figure 33.1), origin, and function, the common name aerolysin will be used throughout this chapter.

## PRODUCTION, PURIFICATION, AND PRIMARY STRUCTURE

Aerolysin from *A. hydrophila* was first discovered and partially purified by Bernheimer and Avigad (Bernheimer *et al*., 1975) and then purified to homogeneity by Buckley and co-workers (Buckley *et al*., 1981). A number of aerolysin genes were cloned and sequenced for different species, including two from *A. hydrophila* (aerolysin, SwissProt entry P09167) (Howard and Buckley, 1986; Howard *et al*., 1987) and Act Q44063 (Chopra *et al*., 1993), one from *A. punctata* (Wang *et al*., 1996) (P94128), *A. trota* (Khan *et al*., 1998) (O85370), and *A. sobria* (Q8GCZ4)(Fujii *et al*., 1998), showing a very high degree of sequence identity at the protein level (Figure 33.1). Interestingly, aerolysin related toxins were also found in a Gram-positive bacterium, *Clostridium septicum* α-toxin (Gordon *et al*., 1997) (Figure 33.1) and in a plant seed enterolobin, (Sousa *et al*., 1994) (Figure 33.1), indicating that the gene crossed not only the Gram-positive/Gram-negative barrier, but also that barrier between prokaryotes and eucaryotes.

Production of high amounts of aerolysin was made possible by expressing the gene in low protease, producing *A. salmonicida* strains allowing purification, using a two-step chromatography of the precursor protein, proaerolysin, from the bacterial supernatant (Buckley, 1990; Chopra *et al*., 2000). Proaerolysin can also be produced in *E. coli* using vectors that allow expression of the protein in the periplasm of the bacterium—to allow disulfide bond formation (see below)—with a C-terminal histidine tag and purification with nickel columns (Tsitrin *et al*., 2002).

## STRUCTURE OF PROAEROLYSIN

The toxin is synthesized as a pre-proaerolysin, with an N-terminal signal sequence that directs it to translocation across the inner bacterial membrane and a C-terminal precursor peptide. The signal sequence is removed upon translocation and the precursor, proaerolysin, is then secreted to the extracellular medium. Proaerolysin is a 470 amino acid long chain, with a molecular mass of approx. 52,000 Da and no hydrophobic stretches, in contrast to most membrane-spanning proteins. When the sequence was obtained, this observation led to the prediction that aerolysin would not cross the membrane as hydrophobic α-helices, but rather as a β-barrel, as do bacterial outer-membrane porins (Weiss *et al*., 1990).

This prediction was supported by the X-ray structure of proaerolysin (Parker *et al*., 1994) (Figure 33.2), which revealed a protein rich in β-structure with little α-helical structure in agreement with circular dichroism data (42% β and 21% α structure) (van der Goot *et al*., 1992). The protein was found to be an L-shaped molecule, divided into a small N-terminal lobe (domain 1) and a large lobe, composed of three additional domains. Two disulfide bonds (C19–C75 in domain 1 and C159–C164 in domain 2) were predicted by the structure, and confirmed experimentally, that stabilize the toxin and protect the 154–167 loop from proteolysis (Lesieur *et al*., 1999).

Proaerolysin crystallized as a head to tail dimer, not surprisingly since dimers can be found in solution (van der Goot *et al*., 1993a; Barry *et al*., 2001) at toxin concentrations above 0.1 mg/ml (Fivaz *et al*., 1999). Dimerization involves a domain-swapping interaction between domain 1 of one monomer and the large lobe of the adjacent monomer. It is not yet clear why dimerization occurs, but presumably it confers stability to the protein or reduces its sensitivity to proteases. Another possibility is that dimerization is required for the secretion of proaerolysin by the bacterium (see below).

Domain 1, the only domain that is continuous in sequence (residues 1–82) plays an important role in receptor binding (through residues Trp-45, Ile-47, Met-57, and Lys-66; Figure 33.2) (MacKenzie *et al*., 1999) and is involved in dimerization. This domain shows a strong structural homology with a fold found in S2 and S3 subunits of pertussis toxin of *Bordetella pertussis* (Rossjohn *et al*., 1997), and similar folds are found in C-type lectins, suggesting a role in carbohydrate binding (see below). Domain 2 (residues 83–178, 311–398) also plays an important role in receptor binding (through residues Tyr-162, Trp-324, His-332; see below) (MacKenzie *et al*., 1999) and additionally is important in the initiation of oligomerization (Buckley *et al*., 1995). Interestingly, domain 2 contains most of the α-helical structure of the protein. Domain 3 (residues 179–195, 224–274, 299–310, and 399–409) is thought to play an important role in monomer-monomer interactions within the oligomer (Lesieur *et al*., 1999). Increasing evidence also points towards a role of domain 3, and in particular one loop, in channel formation as discussed below. Domain 4 (residues 196–233, 275–298, and 410–470) contains the peptide that is proteolytically removed upon conversion of proaerolysin into mature aerolysin and was thought to be responsible for channel formation (Parker *et al*., 1994), a view that has been recently challenged, as discussed below.

Interestingly, the structure of *Clostridium perfringens* epsilon toxin was recently solved (Cole *et al*., 2004).

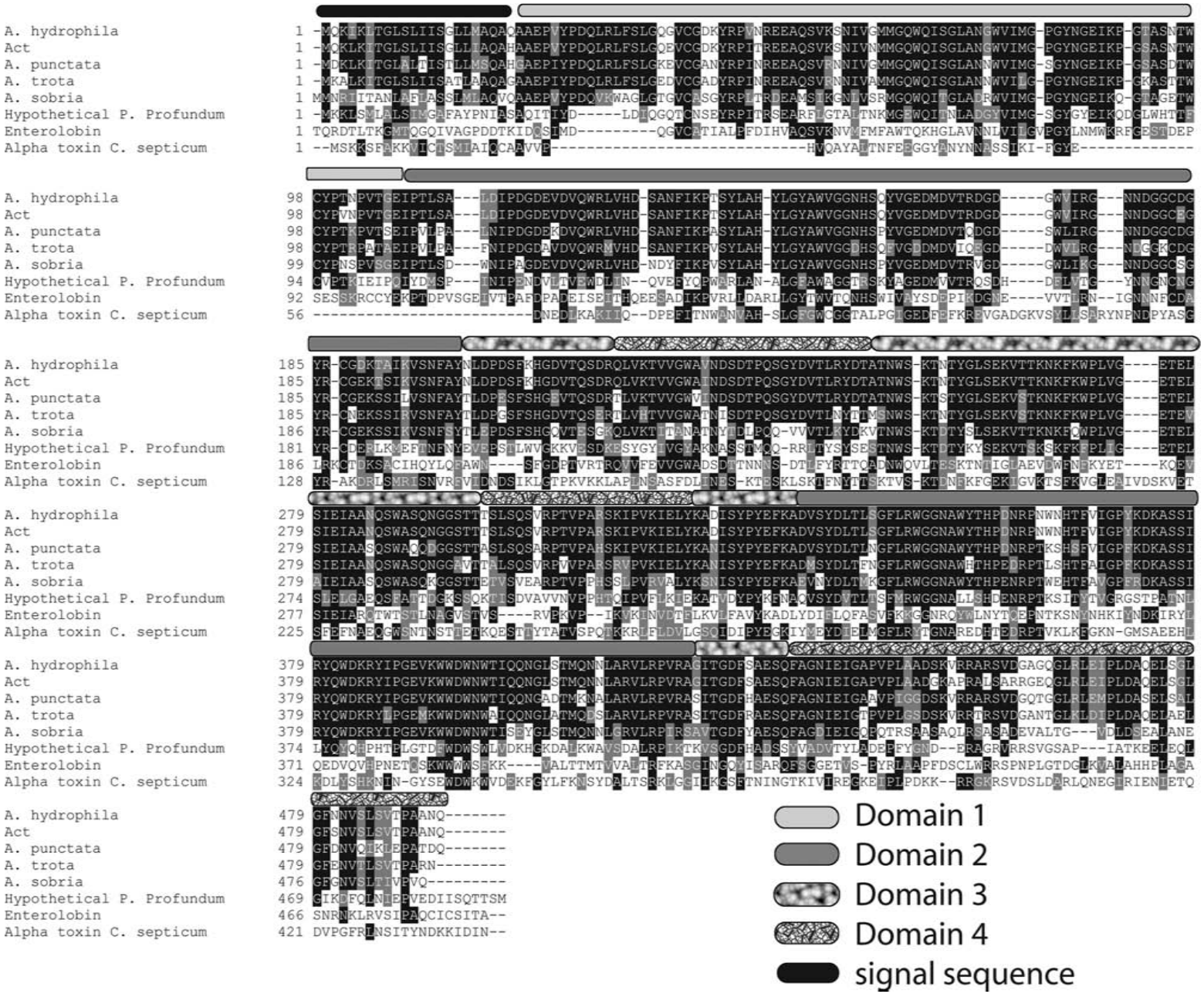



**FIGURE 33.1** Alignment of aerolysins and related toxins. Five different aerolysin-related toxins from four different *Aeromonas* species were aligned to illustrate the extent of sequence identity. A hypothetical protein from *Photobacterium profundum* was also identified when searching for related proteins. As previously shown, aerolysin also shares significant sequence identity with the α-toxin from *Clostridium septicum* and the seed cytolysin enterolobin. The four structural domains of aerolysin, as well as the signal sequence, are indicated in different shades of gray and patterns above the alignment and correspond to the same shades of gray and patterns in Figure 33.2.

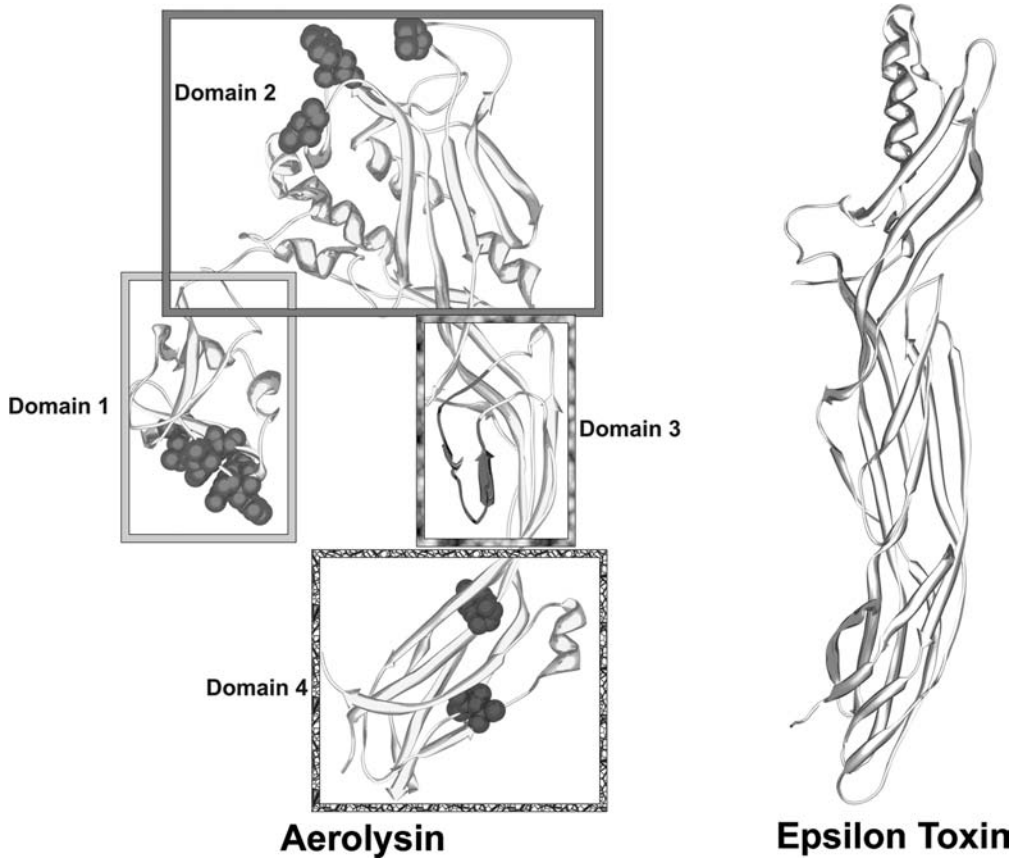



**FIGURE 33.2** Structure of *A. hydrophila* proaerolysin and *C. perfringens* epsilon toxin. Ribbon diagrams are shown to illustrate the structures, and their similarities, of *A. hydrophila* proaerolysin (left) (Parker *et al*., 1994) and *C. perfringens* epsilon toxin (right) (Cole *et al*., 2004). Domains in aerolysin are boxed with shades of gray and patterns corresponding to the alignment in Figure 33.1. Residues that have been implicated in receptor binding are shown in the space filled. In domain 1, these correspond to residues W45, I47, M57, K66 and in domain 2 to residues Y162, W324, H332. They are also shown in the space-filled residue Y221 in domain 3, which when mutated to glycine, inhibits membrane insertion of the toxin, and residues G202 and I445 in domain 4, which when both are mutated to cystein, leads to a disulfide bond between the mature protein and the pro-peptide.

Although this toxin shares only 13.9% identity with aerolysin, it has essentially the same mode of action (proteolytic activation of a precursor, receptor binding, and heptamerization) (Miyata *et al*., 2002), and above all a structure remarkably similar to that of the large aerolysin lobe (Cole *et al*., 2004) (Figure 33.2b), except for the region corresponding to domain 2 of aerolysin. This difference suggests that epsilon toxin might bind to different receptors than aerolysin, a notion that is further supported by the potency of this toxin, which is effective at 100-fold lower concentration and shows a strong cell type specificity.

## SECRETION OF PROAEROLYSIN

Proaerolysin is secreted by *Aeromonas* in a two-step process, which allows it to cross sequentially the inner and then the outer bacterial membrane of the Gram-negative bacterium. Interestingly, complete folding of the protein appears to occur in between the two steps so that the second translocation step occurs in a fully folded state, in contrast to all other known translocation processes, such as crossing the eukaryotic endoplasmic reticulum or mitochondrial membranes.

The N-terminal signal sequence directs the pre-pro-toxin to the *sec* secretion machinery for translocation through the inner bacterial membrane (Howard and Buckley, 1985b; for review see Pugsley, 2004). Within the oxidizing environment of the periplasm, the toxin folds, forms its two disulfide bridges, and can dimerize (Hardie *et al*., 1995), presumably all with the help of periplasmic chaperones and redox folding enzymes. Translocation across the outer bacterial membrane occurs via the so-called type II secretion apparatus, the main branch of the general secretion

pathway (Russel, 1998; Stathopoulos *et al*., 2000). This pathway has been best characterized in *Klebsiella oxytocca* for the secretion of pullulanase (Peabody *et al*., 2003; Pugsley *et al*., 2004) and involves some 14 gene products called the *Pul* proteins in *Klebsiella* and *exe* in *Aeromonas* (Jiang and Howard, 1991, 1992; Howard *et al*., 1993; Jahagirdar and Howard, 1994; Howard *et al*., 1996). The proteins share significant sequence similarity with components of the type IV pilus biogenesis system. Translocation of aerolysin across the outer membrane is poorly understood. It appears that both the electron motive force and ATP are required (Wong and Buckley, 1989; Letellier *et al*., 1997), although there is no known source of ATP available to the outer membrane. The transduction of the metabolic energy to the opening of a secretion port in the outer membrane involves the two inner membrane proteins, ExeA and ExeB (Howard *et al*., 1996). The translocon itself is thought to be composed of the protein ExeD (Ast *et al*., 2002). The *Klebsiella* homologue, PulD, was shown to form homo-oligomeric ring-like pores in reconstituted membranes (Nouwen *et al*., 2000). It is not clear how the gating of such a huge channel would operate and prevent the cell from becoming leaky. The targeting of a protein to this type II secretion system is equally unclear. No specific linear sequence that would target proteins to the type II secretion system has been found, although regions in *Pseudomonas* exotoxin A and in pullulanase have been found to enable secretion of other proteins (Sauvonnet and Pugsley, 1996). The information for secretion through this system might lie in the three-dimensional structure.

## RECEPTOR BINDING

Once in the extracellular milieu, proaerolysin diffuses towards its target cells. Both proaerolysin and mature aerolysin (see below) have the ability to bind specifically to cells. Although the toxin may exist as a dimer, surface binding occurs in the monomeric state (Fivaz *et al*., 1999). Binding to mammalian cells was found to be specific and saturable (Abrami *et al*., 1998b), indicating the existence of a surface receptor. Moreover, no species or cell type specificity was found, suggesting that the receptor was ubiquitous. In the late 1990s, proaerolysin was indeed found to bind to glycosylphosphaditylinositol (GPI) anchored proteins, which are ubiquitous (Cowell *et al*., 1997; Nelson *et al*., 1997; Abrami *et al*., 1998b; Diep *et al*., 1998b). Interestingly, the main binding determinant turned out to be the glycan core of the anchor. This posttranslational modification is present throughout evolution from protozoa to mammals (Kinoshita *et al*., 1997; Ferguson, 1999). GPI-anchored proteins (GPI-AP) have no transmembrane domains, and thus no cytosolic domain. The protein is attached to the cell surface via the GPI anchor, which is composed of a specific phospholipid, phosphatidylinositol (PI), on which are sequentially linked a glucosamine, three mannose residues, and an ethanolamine phosphate, which is linked by an amide bond to the C-terminus of the protein (Figure 33.3a). The inositol ring of the GPI is initially acylated during biosynthesis of the GPI anchor and subsequently deacylated, except in certain cell types such as human erythrocytes (Kinoshita *et al*., 1997; Ferguson, 1999). The conserved GPI glycan core can be additionally modified in a great variety of manners, depending on the species, on any of the different sugars (Kinoshita *et al*., 1997; Ferguson, 1999). Modification of the second mannose by addition of a beta-N-acetylglucosaminyl phosphate diester residue was recently found to be crucial for aerolysin binding (Fukushima *et al*., 2003) (Figure 33.3a). Acquisition of a presynthesized GPI anchor by a newly synthesized protein occurs after the removal, in the endoplasmic reticulum lumen, of a C-terminal GPI-anchoring signal sequence. The lipid-anchored protein is subsequently transported to the plasma membrane.

That GPI-anchored proteins are the receptor of aerolysin is supported by several lines of evidence. Treatment of cells with a phosphatidyl inositol-specific phospholipase C (PI-PLC), which removes the GPI-AP from the surface of the cell, leads to a decrease in sensitivity to aerolysin, except for human erythrocytes, due to acylation of the inositol ring. Also GPI-deficient cells have a three-fold reduced sensitivity to aerolysin (Nelson *et al*., 1997; Gordon, 1999). And finally, screening for aerolysin-resistant Chinese hamster ovary (CHO) mutants led mostly to cells defective in GPI biosynthesis (Abrami *et al*., 2001; Hong *et al*., 2002).

Since the glycan core is the main binding determinant and since it is common to all GPI-anchored proteins, aerolysin is, in principle, able to bind to all GPI-anchored proteins. Indeed, aerolysin was found to bind to a great variety of GPI-anchored proteins that have no sequence homology, such as Thy-1, a major surface glycoprotein of T-lymphocytes and mammalian neurons (Nelson *et al*., 1997); contactin, a brain cell adhesion molecule (Diep *et al*., 1998b); CD14, a monocyte differentiation factor also involved in lipopolysaccharide signaling; semaphorin K1, a brain GPI-anchored protein; carboxypeptidase M (unpublished); and the GPI-anchored form of adhesion molecule N-CAM (Fivaz *et al*., 2002). Two mammalian exceptions, however, have been found: the complement inhibitory protein CD59 (Abrami *et al*., 2002) and

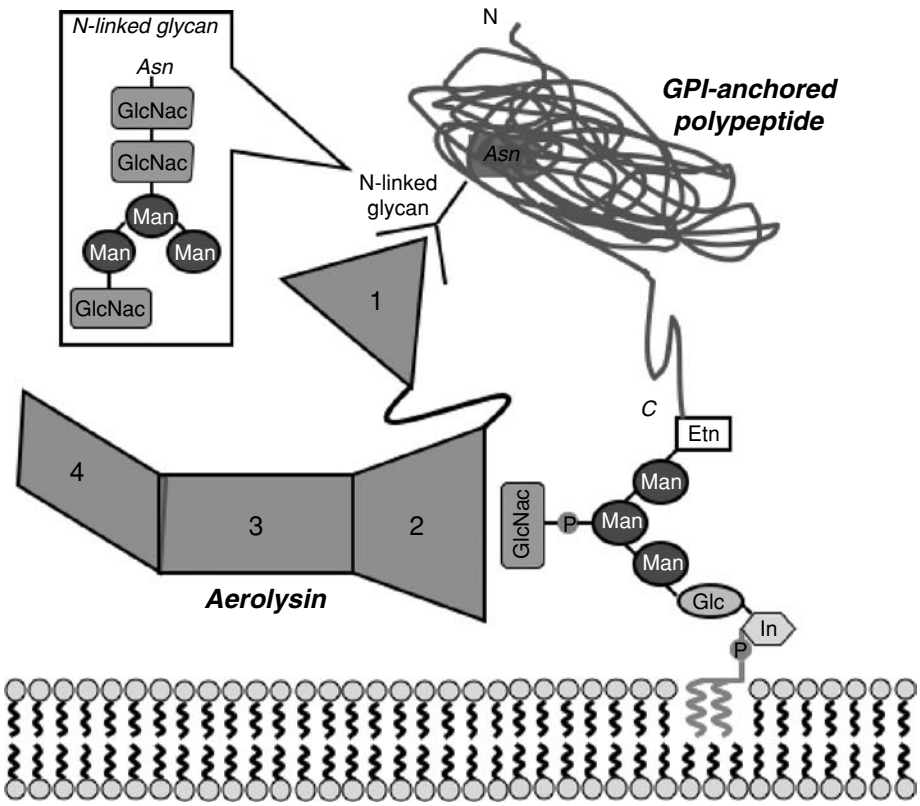



**FIGURE 33.3** Schematic representation of aerolysin bound to its glycosylated GPI-anchored receptors. Aerolysin is shown with its four structural domains. Domain 1 binds to a properly trimmed N-linked sugar, the minimal structure of which is schematized in the inset. Domain 2 binds to the glycan core of the anchor, which is imbedded into the external leaflet of the plasma membrane by a phosphatidylinositol phospholipid. The GPI glycan core shows a GlcNac (N-Acetylglucosamine) modification on the second mannose that is important for receptor binding. Asn: Asparagin, Etn: Ethanol amine, In: inositol, P: phosphate group, Glc: Glucose, Man: Mannose, GlcNac:N-Acetylglucosamine.

the prion protein (unpublished), as well as *Leshmania* gp63 (Abrami *et al*., 2002). This parasite protein was able to bind aerolysin when expressed in mammalian cells (Diep *et al*., 1998b), suggesting that modifications of the glycan core in the parasite hinder aerolysin binding.

Although the identity of the protein attached to the GPI anchor does not influence binding, the presence of the protein was found to be necessary since aerolysin does not bind to protein-free GPIs (Abrami *et al*., 2002). This is because the protein harbors a second binding site for the toxin, as recently found in a screen for low aerolysin binding CHO cells (Hong *et al*., 2002) (Figure 33.3a). Interestingly, as for the first receptor binding site, the toxin does not recognize the polypeptide, but recognizes a second posttranslational modification: the presence of N-linked sugars. ER N-linked glycosylation is not sufficient for aerolysin binding; the N-glycan needs to be properly trimmed. More specifically, α-mannosidase II in the Golgi must remove mannoses from the complex glycan initially added to asparagine residues in the endoplasmic reticulum (Hong *et al*., 2002). Addition of terminal galactose and syalic acid was not required, indicating that the minimum N-glycan structure for aerolysin binding is Gn-M3-Gn2-N (Gn, N-acetylglucosamine; M, mannose; N, asparagine). Since all known GPI-anchored proteins are glycosylated and - often hyperglycosylated, this second binding site does - not reduce the number of potential receptors. Aerolysin shares the requirement of N-linked sugars for binding with *Clostridium botulinum* C2 toxin (Eckhardt *et al*., 2000). This AB type toxin is composed of an enzyme component C2I and a separate binding/translocation component C2II. As aerolysin, C2II undergoes proteolytic activation, heptamerization, and requires asparagine-linked complex glycans for binding to host cells.

To the two binding sites on the aerolysin receptors correspond two binding sites on the toxin, one in domain 1 (Diep *et al*., 1999) and the second in the long lobe of the protein (Diep *et al*., 1998a) (Figures 33.2 and 33.3a). This notion was initially proposed by Buckley and colleagues, based on the observations that a hybrid toxin composed of domain 1 (Figure 32.2) of aerolysin followed by the α toxin of *Clostridium septicum* (Figure 32.1, which is homologous to aerolysin but lacks domain 1), had a higher receptor-binding affinity (approximately 50 times) than α-toxin alone (Diep *et al*., 1999), which also binds GPI-anchored proteins (Gordon *et al*., 1999). More specifically, domain 1 is involved in binding to the

complex N-glycans (Hong *et al*., 2002). Interestingly, recombinant domain 1 appeared to bind only to N-glycans that are present on GPI-anchored proteins, suggesting that it, in fact, recognizes a site to which both the N-glycan and the GPI-glycan core contribute. Mutation of residues Trp-45, Ile-47, Met-57, Tyr-61, and Lys-66 lead to reduced receptor binding as measured by plasmon resonance using immobilized Thy-1 (MacKenzie *et al*., 1999). Binding to the GPI anchor occurs via domain 2, involving in particular Tyr-162, Trp-324, and His-332, which when mutated led to a decrease in Thy-1 binding affinity (MacKenzie *et al*., 1999). The combination of these two binding sites leads to the high-affinity interactions of aerolysin with its receptors. The apparent Kd for binding of proaerolysin to BHK cells was estimated to be around 20nM (Abrami *et al*., 1998b) and similar value was found when measuring binding of proaerolysin to purified Thy-1 using plasmon resonance (66nM) (MacKenzie *et al*., 1999). Strikingly, many of the residues identified as being important for binding to either of the two carbohydrate sites are aromatic residues. Similar observations have been made for other carbohydrate-binding proteins in which aromatic residues have been shown to stack against the pyranose rings of sugars (Quiocho, 1986).

The precise manner in which aerolysin binds to its receptor requires further investigation. Indeed, it is not clear how domain 2, a rather large domain, can interact with a small glycan core, which is sandwiched between the polypeptide of the GPI-anchored protein and the membrane (Figure 33.3). A second puzzle is the binding to N-linked sugars. How can aerolysin bind to almost any GPI-anchored protein despite the fact that these have N-linked sugars at different positions on their tertiary fold? Finally, even more mysterious is how aerolysin reaches its high-affinity binding site on the glycan core of the GPI anchor. Indeed, this part of the receptor is very little exposed at the cell surface, especially in intestinal cells. In order to cross the thick layer of glycocalyx covering epithelial cells, aeolysin may initially bind to surface oligosaccharides with low affinity, possibly through its lectin-like domain 1 or tryptophan-rich regions of the molecule (18 in total), before being progressively shuttled to the membrane vicinity where it would bind to the glycan core of the GPI anchor (MacKenzie *et al*., 1999). This model of relay of low- and high-affinity binding sites remains to be proven, but is supported by the finding that polarized epithelial cells are always more sensitive to aerolysin from their apical side—more abundant in oligosaccharides—than from their basolateral side, irrespective of the localization of their GPI-anchored proteins (Abrami *et al*., 2003).

## FROM THE PRECURSOR TO THE ACTIVE TOXIN

Conversion of proaerolysin to aerolysin requires proteolytic cleavage of a C-terminal peptide (Figure 33.2), a step that can occur prior or subsequent to receptor binding. Cleavage can be achieved by a variety of enzymes either produced by the bacterium (Howard and Buckley, 1985a) or found in the digestive tract, such as trypsin (Howard and Buckley, 1985a), or present at the surface of the cell, such as members of the furin family (Abrami *et al*., 1998a). Three cleavage sites have been identified: Lys-427 by trypsin, Arg-429 by chymotrypsin (van der Goot *et al*., 1992), and Arg-432 by furin (Abrami *et al*., 1998a), suggesting that the loop containing these residues (which was not seen in the crystal structure) is very sensitive to proteases.

Proteolysis at the C-terminus—in domain 4—leads to conformational changes that spread throughout the entire structure all the way to domain 1 (Cabiaux *et al*., 1997). The exact changes have not been identified, but a 10% increase in β-sheet structure at the detriment of random structure was measured by Fourier Transformed Infra Red spectroscopy (FTIR) with no major change in tertiary structure (Cabiaux *et al*., 1997).

## HEPTAMER FORMATION

Once processed into aerolysin and bound to the receptor, the toxin undergoes oligomerization into a ring-like structure composed of seven monomers, as observed both by electron microscopy (Wilmsen *et al*., 1992) and mass spectrometry (Moniatte *et al*., 1996). While oligomerization can occur in solution, it is far more efficient at the surface of cells. This is to a large extent due to the increase in concentration that accompanies the decrease in dimensionality when going from a three- to a two-dimensional space (estimated to be a 3,000- to 10,000-fold increase). An additional concentration factor is provided by the association of the GPI-anchored receptors with special plasma membrane domains, or lipid rafts, thus further promoting oligomerization (Abrami and van der Goot, 1999).

The mechanism by which the heptamer assembles (i.e., does it assemble through gradual association of monomers, or do dimers and trimers form and then associate) has not been characterized. What is known is that oligomerization involves no change in secondary structure, as measured by circular dichroïsm (CD) (van der Goot *et al*., 1993b), but the rearrangement of the tertiary organization as observed both by CD and FTIR (Cabiaux *et al*., 1997). This rearrangement leads to the formation of an extraordinarily stable structure, more

so than heptamers of other toxins, such as staphylococcal α-toxin. The incubation of the aerolysin heptamer in 8M urea for 24 hours not only does not separate the subunits, but does not even affect its tertiary structure as witnessed by circular dichroïsm in the near ultraviolet, which monitors the flexibility of aromatic residues and in particular tryptophans (Lesieur *et al*., 1999). Heptameric assembly is also maintained in guanidium hydrochloride (6M), but this stronger chaotropic agent does lead to loss of tertiary packing. Finally, the heptamer is resistant to boiling in SDS (Lesieur *et al*., 1999).

In order to identify the regions involved in the tight maintenance of the complex, limited proteolysis studies were performed using the enzyme boilysin, an engineered enzyme, which retains its activity at high temperatures and even in the presence of SDS (Van den Burg *et al*., 1998). Boilysin treatment led to truncated but still oligomeric aerolysin complexes, the composition of which was identified by N-terminal sequencing and MALDI-TOF analysis (Lesieur *et al*., 1999). Two fragments, corresponding to amino acids 180–307 and 401–427, were identified, showing that the domains 3 and 4 are crucial for maintaining the heptamers assembled, while domains 1 and 2 are not required (Lesieur *et al*., 1999).

Although domain 2 is not responsible for the extreme stability of the heptamer, it is involved in initiating the oligomerization process. Mutation of His-132 in domain 2 completely abolishes *in vitro* heptamerization (Green and Buckley, 1990) and channel formation in planar lipid bilayers (Wilmsen *et al*., 1991). It was shown that protonation of histidine at position 132 is necessary for oligomerization (Buckley *et al*., 1995). This histidine has a very unusual environment that severely modifies its pKa, allowing histidine protonation at physiological pH. Mutations of His-107 also reduced oligomerization, but to a lesser extent (Green and Buckley, 1990). Mutation of trypthophans at positions 371 and 373, also in domain 2, had the opposite effect since oligomerization was accelerated possibly by a partially destabilizing effect that favors the rearrangements leading to heptamerization (van der Goot *et al*., 1993b).

Many PFTs, including staphylococcal α-toxin and cholesterol-dependent toxins (CDT), first oligomerize into a prepore state and then undergo a second concerted conformational change that leads to channel formation. In the case of aerolysin, there is no evidence for a prepore state, and oligomerization appears to be systematically accompanied by the appearance of hydrophobic patches, as witnessed by measuring binding of fluorescent hydrophobic dyes (van der Goot *et al*., 1993b). A very interesting aerolysin point mutation, Y221G, has been identified that leads to a non-hydrophobic heptamer (Tsitrin *et al*., 2002). This mutant can still bind to GPI-anchored proteins and heptamerize, but these heptamers cannot insert into membranes (Nelson *et al*., 1999; Tsitrin *et al*., 2002). It is not clear whether this mutation allows the trapping of a not previously observed prepore state, whether it leads to aberrant, dead-end heptamers, or whether the mutation, repeated seven times in the heptamer, has converted the hydrophilic complex into a hydrophilic one.

Analysis of the Y221G heptamer by cryoelectron microscopy single particle analysis and 3D reconstruction—rendered possible because of the solubility of the complex—provided a 13 Å model of the aerolysin heptamer, corresponding to a significant improvement over the previous 25 Å resolution model (Wilmsen *et al*., 1992). The aerolysin heptamer has a mushroom-shaped structure (Figure 33.4) in which domains 1 and 2 form the cap, domain 3 the mouth of the channel, and domain 4 the transmembrane part. As described below, experimental evidence suggests that this model is somewhat incorrect, in particular with respect to the positions of domains 3 and 4, despite the rather good fit obtained when docking the crystal structure into the

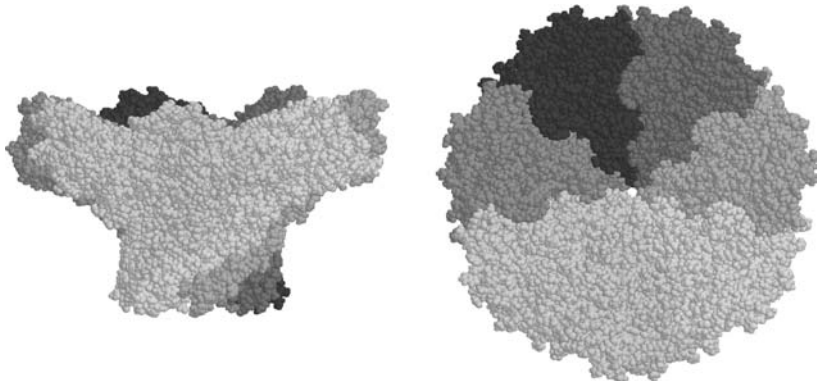


**FIGURE 33.4** Space-filled model of the aerolysin Y221G heptamer. Single particle analysis of soluble Y221G heptamers led to a 3D reconstruction with a 13 Å resolution in which the crystal structure was docked, leading to a high resolution model that is shown here in space-filled, viewed either from the side (left) or from the top (right) (Tsitrin *et al*., 2002).

electron density map (Tsitrin *et al*., 2002). A full understanding of the heptameric structure therefore awaits further studies.

## MEMBRANE INSERTION AND CHANNEL PROPERTIES

Since aerolysin does not appear to transit through a prepore state, heptamerization is directly followed by membrane insertion. As for other β-sheet containing PFTs, channel formation is thought to occur by the formation and spontaneous membrane insertion of a β–barrel. Little is known about this last step, both in terms of the interaction with lipids and the structure of the transmembrane region. It has been suggested that the junction between lipid rafts and the fluid phase region is a site of unfavorable energetic effects, and these unstable boundaries might provide a favorable insertion site (Brown, 1998). Also, the insertion of aerolysin heptamers in artificial liposomes is enhanced by the presence of lipids favoring lamellar-to-inverted hexagonal phase transition (Alonso *et al*., 2000). These authors indeed showed that the rate of dye leakage from unilamellar egg-phosphatidylcholine vesicles is enhanced by the presence of phosphatidylethanolamine, diacylglycerol, or cholesterol. On the other hand, lipids that stabilize the lamellar phase, such as sphingomyelin and saturated phosphatidylcholines, reduced the rate of channel insertion.

It is at present not clear which part of aerolysin actually forms the transmembrane region. The low- and medium-resolution models (Parker *et al*., 1994; Tsitrin *et al*., 2002) of the heptamer propose that domain 4 crosses the membrane. This model is, however, challenged by experiments that indicate that a loop in domain 3 (Figure 33.2) is necessary for channel formation (manuscript in preparation). This loop, corresponding to residues 238 to 260, has previously been implicated in oligomerization since immobilization of this loop, by an engineered disulfide bond that links it to the protein core, inhibited heptamerization (Rossjohn *et al*., 1998). More recently, we have found that restraining the flexibility of the loop, by disulfide bond engineering within the loop, does not prevent heptamerization but blocks channel formation (in preparation). Finally, the corresponding loop in *C. Septicum* α-toxin was also found to interact with the membrane (Melton *et al*., 2004). Formal proof that the loop actually crosses the bilayer is still lacking, however. If, indeed, the loop does form the transmembrane channel, the role and localization of domain 4 will have to be seriously reevaluated and the discrepancy between the function data and the electron microscopy analysis elucidated.

Electrophysiological measurements in planar lipid bilayers showed that aerolysin makes well-defined, slightly anion selective channels with a single-channel conductance of 420pS (Chakraborty *et al*., 1990; Wilmsen *et al*., 1990). The channels remain opened in the range of –70 to +70 mV and undergo voltage-dependent closing outside of this range. From the analysis of 2D crystals of the aerolysin heptamers, the measured inner diameter of the channel was estimated at 17 Å (Parker *et al*., 1994; Tsitrin *et al*., 2002), but somewhat greater diameters were reported from small-molecule release experiments (30 Å) (Howard and Buckley, 1982) and optical single-channel analysis (40 Å) (Tschödrich-Rotter *et al*., 1996). *In vivo* data suggests that the channels formed in living cells are somewhat smaller, as the heptamer seems to discriminate between ethidium monomer and ethidium dimer (Krause *et al*., 1998) and does not allow the passage of entire molecules of trypan blue (960 Da) into cells (Abrami *et al*., 1998b).

## CELLULAR CONSEQUENCES OF AEROLYSIN

Upon channel formation in the plasma membrane of mammalian cells, aerolysin leads to the permeabilization to small ions but not proteins (Abrami *et al*., 1998b). As the pore allows ions to go through, this leads to a dissipation of the $Na^+$ and $K^+$ gradients and the loss of membrane potential (Abrami *et al*., 1998b; Laohachai *et al*., 2003). Importantly, aerolysin channels also allow the entry of extracellular calcium (Krause *et al*., 1998).

In cells that are unable to repair or counteract membrane damage, this breach in plasma membrane permeability leads to osmotic lysis. However, except at late stages of *Aeromonas* infection when a bacteremia is reached, the toxin is unlikely to encounter erythrocytes. In nucleated cells, the consequences of membrane damage by aerolysin are far more complex and are both concentration- and cell-type dependent. We will briefly describe some of the observed effects. The link between these various observations remains unclear, and obtaining the full picture will require intensive further studies. The massive efflux of potassium and the entry of calcium are two events that on their own are likely to cause a multitude of effects.

### Morphological changes

At concentrations of 1 nM and below, intestinal and other epithelial cells undergo cytoplasmic vacuolation. These vacuoles were found to originate from the endo-

plasmic reticulum, leaving the endocytic compartments and Golgi complex morphologically unchanged (Abrami *et al*., 1998a; Abrami *et al*., 1998b). This process was inhibited when extracellular calcium was chelated with EGTA (unpublished), but was not solely dependent on calcium entry since calcium ionophores did not lead to ER vacuolation (Abrami *et al*., 1998b). ER vacuolation was dependent on the dynamic properties of the compartment since vacuolation was inhibited by ATP depletion or depolymerization of the microtubules network (Abrami *et al*., 1998b). Induction of neutral pH cytoplasmic vacuoles was also observed for other pore-forming toxins, such as Staphylococcal α-toxin (Abrami *et al*., 1998b), *Serratia marcescens* hemolysin (Hertle *et al*., 1999), and *Vibrio cholera* hemolysin (Coelho *et al*., 2000; Mitra *et al*., 2000), although in the two latter cases, the ER was not formally identified. ER vacuolation was not observed for Streptolysin O, possibly because it forms larger pores (Walev *et al*., 2001). Note that these PFT-induced vacuoles differ from those induced by the *Helicobacter pylori* vacuolating toxin VacA, which leads to acidic vacuoles that originate from late endocytic compartments (Montecucco and de Bernard, 2003).

In addition to the appearance of cytoplasmic vacuoles, intestinal and epithelial cells often undergo plasma membrane blebbing when exposed to aerolysin (Abrami *et al*., 1998a; Abrami *et al*., 1998b; Galindo *et al*., 2004b).

## Changes in gene expression and production in pro-inflamatory molecules

Chopra and colleagues have extensively analyzed the changes in gene expression upon exposure to aerolysin, both of RAW macrophages and of primary macrophages (Chopra *et al*., 2000; Galindo *et al*., 2003; Galindo *et al*., 2004a). These studies have led to the identification of some 200 up- and 200 down-regulated genes between 0 and 12 h of toxin treatment. Further studies will be required to understand how these genes fall into pathways and what their relevance is. Immediately apparent, however, and perhaps not surprisingly, aerolysin was found to trigger the production of a variety of pro-inflammatory molecules, in particular tumor necrosis factor α (TNFα), interleukin 1β (ILlβ), interleukin 6, and protasglandin E2 (Chopra *et al*., 2000; Ribardo *et al*., 2002; Galindo *et al*., 2003; Galindo *et al*., 2004a). Aerolysin was also found to induce the expression of nitric oxyde synthase (iNOS), possibly via up-regulation of TNF-α and IL-1 in the host cell (Chopra *et al*., 2000). Nitric oxyde (NO) production by iNOS is an essential element of antimicrobial immunity and host-induced tissue damage.

## Induction of apoptosis

That aerolysin triggers apoptosis was first observed on T lymphocytes, as witnessed by DNA laddering, an event that could be overcome by the overexpression of bcl2 (Nelson *et al*., 1999). Since then, aerolysin also has been shown to trigger apoptosis in murine macrophages (Galindo *et al*., 2003) and intestinal cells (Galindo *et al*., 2004b), but not on fibroblast-like cells (Abrami *et al*., 1998b). Note, however, that different assays were used by different laboratories to monitor apoptosis, including DNA condensation and genomic DNA laddering, annexin V binding.

The pathway(s) by which aerolysin triggers apoptosis has not been elucidated. Entry of calcium through the aerolysin channel has been proposed to be the cause (Nelson *et al*., 1999). A recent study suggests that apoptosis could be a secondary effect due to the toxin-induced production of TNFα (Galindo *et al*., 2004b). Galindo *et al*. observed that apoptosis, and the concurrent cleavage of caspase 3, was drastically reduced in aerolysin-treated macrophage lacking the two TNF receptors TNFR-1 and TNFR-2 (Galindo *et al*., 2004b).

## Activation of signaling pathways

The first evidence that aerolysin was able to trigger signaling cascades came from the observation that channel formation led to the activation of Pertussis-sensitive G proteins that subsequently led to the production of inositol (1,4,5)-triphosphate (IP3) and the release of calcium from the ER via IP3-dependent channels (Krause *et al*., 1998). It was apparent from this study that increases in cytoplasmic calcium were not only due to entry of the ion through the toxin channel at the plasma membrane, but also that the toxin triggered release from intracellular stores. Intracellular calcium release was clearly dependent upon pore formation, and not binding or clustering of GPI-anchored proteins, since channel-impaired mutants (such as Y221G or G202C-I445C) had no effect. How aerolysin is able to trigger activation of G proteins remains mysterious, but could involve stretch- or stress-activated receptors. Another possibility is that the aerolysin channels affect the integrity of rafts, which have been implicated in modulating and integrating signaling events at the plasma membrane (Simons and Toomre, 2000). In agreement with the activation of G-proteins, aerolysin was found to stimulate chemotaxis (Jin *et al*., 1992; Krause *et al*., 1998).

In addition to G proteins, aerolysin triggers the activation of multiple signaling cascades. Activation of p38, ERK1/2, and JNK kinases, as well as some of their upstream effectors and downstream targets, has been

observed (Galindo *et al*., 2004b), indicating that aerolysin activates three of the major MAPK signaling routes present in cells involved in cell proliferation, differentiation, apoptosis, and inflammation. The relevance of these findings remains to be fully evaluated.

### Evidence that cells mount a defense against aerolysin

The fact that perforation of the plasma membrane triggers a great variety of effects in the host cell, leading to the up- and down-regulation of genes, is not so surprising, but thorough analysis of the mechanisms involved is now required.

Most astonishing is the recent finding that cells, and organisms, actually mount a defense against pore-forming toxins. The observation was originally made by gene expression analysis of *Caenorhabditis elegans* challenged with an insecticidal, pore-forming toxin (Huffman *et al*., 2004). As for aerolysin-treated macrophages, pore-forming toxins treated with *C. elegans* led to the up- and down-regulation of a variety of genes, among which was the *C. elegans* homologue of p38 MAPK. Aorian and colleagues subsequently showed that worms harboring a mutation in this gene were hypersensitive to the toxin (Huffman *et al*., 2004). These studies were extended to mammalian cells and aerolysin and indicated that cells treated with a p38 MAPK inhibitor died more rapidly than control cells (Huffman *et al*., 2004). This is the first time that a survival pathway is observed as being activated and functional against PFTs. The survival strategy of cells, linked to the innate immune system, is likely to include other pathways, which remain to be found.

## AEROLYSIN AS A TOOL

### Study of GPI-anchored proteins

Mammalian GPI-anchored proteins, which all contain a similar, if not the same, glycan core and are generally highly glycosylated, respond to the two requirements for efficient aerolysin binding. With the exception of two mammalian proteins [CD59 (Abrami *et al*., 2002) and the prion protein], aerolysin does not appear to discriminate between GPI-anchored proteins and is therefore a powerful tool to identify, purify, and study them.

Using aerolysin overlay assays (Cowell *et al*., 1997; Abrami *et al*., 1998b) and two-dimensional electrophoresis, the map of all GPI-APs in a given cell type can be generated (Fivaz *et al*., 2000; Fivaz *et al*., 2002), which combined to the mass spectrometry can lead to their identification (Fivaz *et al*., 2000). Such studies have revealed that cells only express a specific set of GPI-AP, which greatly varies from one cell type to the other. For example, 10 different GPI-linked proteins could be detected on baby hamster kidney cells, whereas rat R6 cells only produced a single GPI-anchored protein, which is Thy-1. Aerolysin overlays are and have also been useful to probe for GPI-anchored proteins on one-dimensional gels of whole cell extracts or subcellular fractions (Drab *et al*., 2001; Herreros *et al*., 2001).

In addition to its use as a pan-GPI–anchored protein probe in biochemical assay, aerolysin and its inactive mutants can be used as probe for both electron (Fivaz *et al*., 2002) and fluorescence microscopy on both fixed and live cells (Abrami *et al*., 2002; Fivaz *et al*., 2002; Sotgia *et al*., 2002; Sharma *et al*., 2004). A useful mutant for detection of GPI-anchored proteins with aerolysin on living cells is the so-called ASSP in which the activation peptide is linked to the mature toxin by an engineered disulfide bond, and therefore remains inactive unless it is reduced, a step that occurs upon arrival in late endocytic compartments (Fivaz *et al*., 2002). This probe binds as a monomer to its receptor and therefore does not lead to clustering. In contrast, the Y221G mutant is a single-point mutation that does not hinder heptamerization but prevents membrane insertion (Tsitrin *et al*., 2002), making it a useful probe to induced controlled clustering of GPI-anchored proteins (Fivaz *et al*., 2002; Sharma *et al*., 2004) in contrast to antibodies.

### Future applications of aerolysin as a probe to study GPI-anchored proteins

One interesting example is the use of aerolysin and its mutants to coat atomic force microscopy cantilevers to study the surface behavior of these lipid-anchored molecules and their surrounding environment (Kasas, van der Goot and Lafont, in preparation).

### Diagnostic

The ability of aerolysin to bind to GPI-anchored proteins has also made it a powerful tool to diagnose a disease that is due to the absence of this class of proteins on hematopoietic cells, namely paroxysmal nocturnal hemoglobinuria (PNH) (Brodsky *et al*., 1999; Brodsky *et al*., 2000). PNH is a clonal stem cell disorder caused by a somatic mutation of the pig-A gene, which is implicated in the biosynthetic pathway of GPI-anchored proteins (Kinoshita *et al*., 1997). PNH blood cells (erythrocytes, lymphocytes, and granulocytes), in contrast to blood cells from healthy patients,

were found to be resistant to aerolysin. So far, the current diagnosis for PNH had been flow cytometric analysis of surface CD59, a GPI-AP implicated in the protection against complement lysis. Aerolysin is, however, a far more sensitive tool to detect PNH populations, in addition to the fact that it is a far simpler and less expensive test (Krauss, 2003; Wang *et al*., 2003).

### Other applications

Aerolysin has also been shown to be useful for purification of parasites, such as trypanosomes and Leishmania, from tissue or blood. These parasites are completely insensitive to the toxin, in contrast to the host blood cells, thereby allowing lysis of the mammalian cells and purification of the parasite (Pearson *et al*., 1982). Considering that these parasites are covered with GPI-anchored proteins, i.e., potential aerolysin receptors, this finding seems surprising. GPI-anchored proteins may be so densely packed at the surface of the parasites (Ferguson, 1999) that aerolysin does not have access to its binding site on the glycan core of the anchor. For Leishmania, another explanation is that *in vitro*, aerolysin is not able to bind to the leishmainia GPI-AP gp-63, suggesting that the structure of the anchor is not compatible with aerolysin binding (Abrami *et al*., 2002).

## CONCLUSION

Tremendous progress has been made over the last decade in the understanding of the mode of action of aerolysin: the structure of proaerolysin has been solved, the receptors have been identified, and two different steps in host cell interaction have been identified. The next period should bring our understanding to a more atomic level: How does oligomerization take place, how does membrane insertion occur, and what is the structure of the final channel? The coming years should also bring a clearer picture as to how a cell reacts to pore formation—how is the breach in the plasma membrane sensed? Are membrane repair mechanisms turned on? Is there a branch of the innate immune system that is able to counteract pore-forming toxins?

## ACKNOWLEDGMENTS

We would like to thank Laurence Abrami for the critical reading of this chapter. This work was supported by the Swiss National Science Foundation.

## REFERENCES

Abrami, L., Fivaz, M., Decroly, E., Seidah, N.G., François, J., Thomas, G., Leppla, S., Buckley, J.T. and van der Goot, F.G. (1998a). The pore-forming toxin proaerolysin is processed by furin. *J. Biol. Chem*. **273,** 32656–32661.

Abrami, L., Fivaz, M., Glauser, P.-E., Parton, R.G. and van der Goot, F.G. (1998b). A pore-forming toxin interacts with a GPI-anchored protein and causes vacuolation of the endoplasmic reticulum. *J. Cell Biol*. **140,** 525–540.

Abrami, L., Fivaz, M., Glauser, P.-E., Sugimoto, N., Zurzolo, C. and van der Goot, F.G. (2003). Sensitivity of polarized epithelial cells to the pore-forming toxin aerolysin. *Infect. Immun.* (in press).

Abrami, L., Fivaz, M., Kobayashi, T., Kinoshita, T., Parton, R.G. and van der Goot, F.G. (2001). Cross-talk between caveolae and glycosylphosphatidylinositol-rich domains. *J. Biol. Chem*. **276,** 30729–30736.

Abrami, L. and van der Goot, F.G. (1999). Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. *J. Cell Biol*. **147,** 175–184.

Abrami, L., Velluz, M.-C., Hong, H., Ohishi, K., Mehlert, A., Ferguson, Kinoshita, T. and van der Goot, F.G. (2002). The glycan core of GPI-anchored proteins modulates aerolysin binidng but is not sufficient: the polypeptide moiety is required for the toxin-receptor interaction. *FEBS Lett*. **512,** 249–254.

Alonso, A., Goni, F.M. and Buckley, J.T. (2000). Lipids favoring inverted phase enhance the ability of aerolysin to permeabilize liposome bilayers. *Biochemistry* **39,** 14019–14024.

Altwegg, M. and Geiss, H.K. (1989). Aeromonas as a human pathogen. *Crit. Rev. Microbiol*. **16,** 253–286.

Ast, V.M., Schoenhofen, I.C., Langen, G.R., Stratilo, C.W., Chamberlain, M.D. and Howard, S.P. (2002). Expression of the ExeAB complex of Aeromonas hydrophila is required for the localization and assembly of the ExeD secretion port multimer. *Mol. Microbiol*. **44,** 217–231.

Austin, B., Altweg, M., Gosling, P.J. and Joseph, S.W. (1996). The genus *Aeromonas*. John Wiley: Chichester.

Barry, R., Moore, S., Alonso, A., Ausio, J. and Buckley, J.T. (2001). The channel-forming protein proaerolysin remains a dimer at low concentrations in solution. *J. Biol. Chem*. **276,** 551–554.

Bernheimer, A., Avigad, L. and Avigad, G. (1975). Interactions between aerolysin, erythrocytes, and erythrocyte membranes. *Infect. Immun*. **11,** 1312–1319.

Brodsky, R.A., Mukhina, G.L., Li, S., Nelson, K.L., Chiurazzi, P.L., Buckley, J.T. and Borowitz, M.J. (2000). Improved detection and characterization of paroxysmal nocturnal hemoglobinuria using fluorescent aerolysin. *Am. J. Clin. Pathol*. **114,** 459–466.

Brodsky, R.A., Mukhina, G.L., Nelson, K.L., Lawrence, T.S., Jones, R.J. and Buckley, J.T. (1999). Resistance of paroxysmal nocturnal hemoglobinuria cells to the glycosylphosphatidylinositol-binding toxin aerolysin. *Blood* **93,** 1749–1756.

Brown, R.E. (1998). Sphingolipid organization: what physical studies of model membranes reveal. *J. Cell Sci*. **111,** 1–9.

Buckley, J.T. (1990). Purification of cloned proaerolysin released by a low protease mutant of *Aeromonas salmonicida. Bioch. Cell Biol*. **68,** 221–224.

Buckley, J.T., Halasa, L.N., Lund, K.D. and MacIntyre, S. (1981). Purification and some properties of the hemolytic toxin aerolysin. *Can. J. Biochem* **59,** 430–435.

Buckley, J.T., Wilmsen, H.U., Lesieur, C., Schultze, A., Pattus, F., Parker, M.W. and van der Goot, F.G. (1995). Protonation of His-132 promotes oligomerization of the channel-forming toxin Aerolysin. *Biochemistry* **34,** 16450–16455.

Cabiaux, V., J.T., B., Wattiez, R., Ruysschaert, J.-M., Parker, M.W. and van der Goot, F.G. (1997). Conformational changes in aerolysin

during the transition from the water-soluble protoxin to the membrane channel. *Biochemistry* **36,** 15224–15232.

Chakraborty, T., Huhle, B., Berghauer, H. and Goebel, W. (1987). Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated infections. *Infect. Immun*. **55,** 2274–2280.

Chakraborty, T., Schmid, A., Notermans, S. and Benz, R. (1990). Aerolysin of aeromonas-sobria—evidence for formation of ion-permeable channels and comparison with alpha-toxin of staphylococcus-aureus. *Infec. Immun*. **58,** 2127–2132.

Chopra, A.K., Houston, C.W., Peterson, J.W. and Jin, G.F. (1993). Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from Aeromonas hydrophila. *Can. J. Microbiol*. **39,** 513–523.

Chopra, A.K., Xu, X., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J.W. and Houston, C.W. (2000). The cytotoxic enterotoxin of Aeromonas hydrophila induces pro-inflammatory cytokine production and activates arachidonic acid metabolism in macrophages. *Infect. Immun*. **68,** 2808–2818.

Coelho, A., Andrade, J.R., Vicente, A.C. and Dirita, V.J. (2000). Cytotoxic cell-vacuolating activity from *Vibrio cholerae* hemolysin. *Infect. Immun*. **68,** 1700–1705.

Cole, A.R., Gibert, M., Popoff, M., Moss, D.S., Titball, R.W. and Basak, A.K. (2004). Clostridium perfringens epsilon-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat. Struct. Mol. Biol*. **11,** 797–798.

Cowell, S., Aschauer, W., Gruber, H.J., Nelson, K.L. and Buckley, J.T. (1997). The erythrocyte receptor for the channel-forming toxin aerolysin is a novel glycosylphosphatidylinositol-anchored protein. *Mol. Microbiol*. **25,** 343–350.

Diep, D.B., Lawrence, T.S., Ausio, J., Howard, P. and Buckley, J.T. (1998a). Secretion and properties of the large and small lobes of the channel-forming toxin aerolysin. *Mol. Microbiol*. **30,** 341–352.

Diep, D.B., Nelson, K.L., Lawrence, T.S., Sellman, B.R., Tweten, R.K. and Buckley, J.T. (1999). Expression and properties of an aerolysin–Clostridium septicum alpha toxin hybrid protein. *Mol. Microbiol*. **31,** 785–794.

Diep, D.B., Nelson, K.L., Raja, S.M., McMaster, R.W. and Buckley, J.T. (1998b). Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin Aerolysin. *J. Biol. Chem*. **273,** 2355–2360.

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F.C., Schedl, A., Haller, H. and Kurzchalia, T.V. (2001). Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* **293,** 2449–2452.

Eckhardt, M., Barth, H., Blocker, D. and Aktories, K. (2000). Binding of Clostridium botulinum C2 toxin to asparagine-linked complex and hybrid carbohydrates. *J. Biol. Chem*. **275,** 2328–2334.

Ferguson, M.A.J. (1999). The structure, biosynthesis, and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. *J. Cell Sci*. **112,** 2799–2809.

Fivaz, M., Velluz, M.C. and van Der Goot, F.G. (1999). Dimer dissociation of the pore-forming toxin aerolysin precedes receptor binding. *J. Biol. Chem*. **274,** 37705–37708.

Fivaz, M., Vilbois, F., Pasquali, C. and van der Goot, F.G. (2000). Analysis of GPI-anchored proteins by two-dimensional gel electrophoresis. *Electrophoresis* **21,** 3351–3356.

Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P., Parton, R. and van der Goot, F. (2002). Differential sorting and fate of endocytosed GPI-anchored proteins. *EMBO. J*. **21,** 3989–4000.

Fujii, Y., Nomura, T., Kanzawa, H., Kameyama, M., Yamanaka, H., Akita, M., Setsu, K. and Okamoto, K. (1998). Purification and characterization of enterotoxin produced by Aeromonas sobria. *Microbiol. Immunol*. **42,** 703–714.

Fukushima, K., Ikehara, Y., Kanai, M., Kochibe, N., Kuroki, M. and Yamashita, K. (2003). A beta-N-acetylglucosaminyl phosphate diester residue is attached to the glycosylphosphatidylinositol anchor of human placental alkaline phosphatase: a target of the channel-forming toxin aerolysin. *J. Biol. Chem*. **278,** 36296–36303.

Galindo, C.L., Fadl, A.A., Sha, J. and Chopra, A.K. (2004a). Microarray analysis of Aeromonas hydrophila cytotoxic enterotoxin-treated murine primary macrophages. *Infect. Immun*. **72,** 5439–5445.

Galindo, C.L., Fadl, A.A., Sha, J., Gutierrez, C., Jr., Popov, V.L., Boldogh, I., Aggarwal, B.B. and Chopra, A.K. (2004b). Aeromonas hydrophila cytotoxic enterotoxin activates mitogen-activated protein kinases and induces apoptosis in murine macrophages and human intestinal epithelial cells. *J. Biol. Chem*. **279,** 37597–37612.

Galindo, C.L., Sha, J., Ribardo, D.A., Fadl, A.A., Pillai, L. and Chopra, A.K. (2003). Identification of Aeromonas hydrophila cytotoxic enterotoxin-induced genes in macrophages using microarrays. *J. Biol. Chem*. **278,** 40198–40212.

Gordon, V.M., Benz, R., Fujii, K., Leppla, S.H. and Tweten, R.K. (1997). Clostridium septicum alpha-toxin is proteolytically activated by furin. *Infect. Immun*. **65,** 4130–4134.

Gordon, V.M., Nelson, K.L., Buckley, J.T., Stevens, V.L., Tweten, R.K., Elwood, P.C. and Leppla, S.H. (1999). Clostridium septicum alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. *J. Biol. Chem*. **274,** 27274–27280.

Green, M.J. and Buckley, J.T. (1990). Site-directed mutagenesis of the hole-forming toxin aerolysin—studies on the roles of histidines in receptor binding and oligomerization of the monomer. *Biochemistry* **29,** 2177–2180.

Hardie, K.R., Schulze, A., Parker, M.W. and Buckley, J.T. (1995). Vibrio sp. secrete proaerolysin as a folded dimer without the need for disulfide bond formation. *Molecular Microbiology* **17,** 1035–1044.

Herreros, J., Ng, T. and Schiavo, G. (2001). Lipid rafts act as specialized domains for tetanus toxin binding and internalization into neurons. *Mol. Biol. Cell* **12,** 2947– 2960.

Hertle, R., Hilger, M., Weingardt-Kocher, S. and Walev, I. (1999). Cytotoxic action of Serratia marcescens hemolysin on human epithelial cells. *Infect. Immun*. **67,** 817–825.

Hong, Y., Ohishi, K., Inoue, N., Kang, J.Y., Shime, H., Horiguchi, Y., van der Goot, F.G., Sugimoto, N. and Kinoshita, T. (2002). Requirement of N-glycan on GPI-anchored proteins for efficient binding of aerolysin but not Clostridium septicum a-toxin. *EMBO. J*. **21,** 5047–5056.

Howard, S.P. and Buckley, J.T. (1982). Membrane glycoprotein receptor and hole-forming properties of a cytolytic protein toxin. *Biochemistry* **21,** 1662–1667.

Howard, S.P. and Buckley, J.T. (1985a). Activation of the hole-forming toxin aerolysin by extracellular processing. *J. Bacteriol*. **163,** 336–340.

Howard, S.P. and Buckley, J.T. (1985b). Protein export by a Gram-negative bacterium: production of aerolysin by Aeromonas hydrophila. *J. Bacteriol*. **161,** 1118–1124.

Howard, S.P. and Buckley, J.T. (1986). Molecular cloning and expression in Escherichia coli of the structural gene for the hemolytic toxin aerolysin from Aeromonas hydrophila. *Mol. Gen. Genet*. **204,** 289–295.

Howard, S.P., Critch, J. and Bedi, A. (1993). Isolation and analysis of eight exe genes and their involvement in extracellular protein secretion and outer membrane assembly in Aeromonas hydrophila. *J. Bacteriol*. **175,** 6695–6703.

Howard, S.P., Garland, W.J., Green, M.J. and Buckley, J.T. (1987). Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *J. Bacteriol*. **169,** 2869–2871.

Howard, S.P., Meiklejohn, H.G., Shivak, D. and Jahagirdar, R. (1996). A TonB-like protein and a novel membrane protein containing an ATP–binding cassette function together in exotoxin secretion. *Mol. Microbiol*. **22,** 595–604.

Huffman, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, F.G. and Aroian, R.V. (2004). Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. *Proc. Natl. Acad. Sci. USA* **101,** 10995–11000.

Jahagirdar, R. and Howard, S.P. (1994). Isolation and characterization of a second exe operon required for extracellular protein secretion in Aeromonas hydrophila. *J. Bacteriol*. **176,** 6819–6826.

Janda, J.M. and Abbott, S.L. (1998). Evolving concepts regarding the genus Aeromonas: an expanding Panorama of species, disease presentations, and unanswered questions. *Clin. Infect. Dis*. **27,** 332–344.

Jiang, B. and Howard, S.P. (1991). Mutagenesis and isolation of aeromonas-hydrophila genes which are required for extracellular secretion. *J. Bacteriol*. **173,** 1241–1249.

Jiang, B. and Howard, S.P. (1992). The aeromonas-hydrophila exeE Gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. *Mol. Microbiol*. **6,** 1351–1361.

Jin, G.-F., Chopra, A.K. and Houston, C.W. (1992). Stimulation of neutrophil leukocyte chemotaxis by a cloned cytolytic enterotoxin of *Aeromonas hydrophila. FEMS Microbiol. Lett*. **98,** 285–290.

Khan, A.A., Kim, E. and Cerniglia, C.E. (1998). Molecular cloning, nucleotide sequence, and expression in Escherichia coli of a hemolytic toxin (aerolysin) gene from Aeromonas trota. *Appl. Environ. Microbiol*. **64,** 2473–2478.

Kingombe, C.I., Huys, G., Tonolla, M., Albert, M.J., Swings, J., Peduzzi, R. and Jemmi, T. (1999). PCR detection, characterization, and distribution of virulence genes in Aeromonas spp. *Appl. Environ. Microbiol*. **65,** 5293–5302.

Kinoshita, T., Ohishi, K. and Takeda, J. (1997). GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. *J. Biochem*. (Tokyo) **122,** 251–257.

Kirov, S.M. (1997). Aeromonas and plesiomonas species. *American Society for Microbiology:* Washington DC.

Krause, K.H., Fivaz, M., Monod, A. and van der Goot, F.G. (1998). Aerolysin induces G-protein activation and Ca2+ release from intracellular stores in human granulocytes. *J. Biol. Chem*. **273,** 18122–18129.

Krauss, J.S. (2003). Laboratory diagnosis of paroxysmal nocturnal hemoglobinuria. *Ann. Clin. Lab. Sci*. **33,** 401–406.

Laohachai, K.N., Bahadi, R., Hardo, M.B., Hardo, P.G. and Kourie, J.I. (2003). The role of bacterial and non-bacterial toxins in the induction of changes in membrane transport: implications for diarrhea. *Toxicon* **42,** 687–707.

Lesieur, C., Frutiger, S., Hughes, G., Kellner, R., Pattus, F. and van Der Goot, F.G. (1999). Increased stability upon heptamerization of the pore-forming toxin aerolysin. *J. Biol. Chem*. **274,** 36722–36728.

Letellier, L., Howard, S.P. and Buckley, J.T. (1997). Studies on the energetics of proaerolysin secretion across the outer membrane of Aeromonas species. Evidence for a requirement for both the protonmotive force and ATP. *J. Biol. Chem*. **272,** 11109–11113.

MacKenzie, C.R., Hirama, T. and Buckley, J.T. (1999). Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. *J. Biol. Chem*. **274,** 22604–22609.

Melton, J.A., Parker, M.W., Rossjohn, J., Buckley, J.T. and Tweten, R.K. (2004). The identification and structure of the membrane-spanning domain of the Clostridium septicum alpha toxin. *J. Biol. Chem*. **279,** 14315–14322.

Mitra, R., Figueroa, P., Mukhopadhyay, A.K., Shimada, T., Takeda, Y., Berg, D.E. and Nair, G.B. (2000). Cell vacuolation, a manifestation of the El tor hemolysin of vibrio cholerae. *Infect. Immun*. **68,** 1928–1933.

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S. and Okabe, A. (2002). Clostridium perfringens epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. *J. Biol. Chem*. **277,** 39463–39468.

Moniatte, M., van der Goot, F.G., Buckley, J.T., Pattus, F. and Van Dorsselaer, A. (1996). Characterization of the heptameric pore-forming complex of the *Aeromonas* toxin aerolysin using MALDI-TOF mass spectrometry. *FEBS Lett*. **384,** 269–272.

Montecucco, C. and de Bernard, M. (2003). Molecular and cellular mechanisms of action of the vacuolating cytotoxin (VacA) and neutrophil-activating protein (HP-NAP) virulence factors of Helicobacter pylori. *Microbes. Infect*. **5,** 715–721.

Nelson, K.L., Brodsky, R.A. and Buckley, J.T. (1999). Channels formed by subnanomalar concentrations of the toxin aerolysin trigger apoptosis of T lymphomas. *Cell. Microbiol*. **1,** 69–74.

Nelson, K.L., Raja, S.M. and Buckley, J.T. (1997). The GPI-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *J. Biol. Chem*. **272,** 12170–12174.

Nouwen, N., Stahlberg, H., Pugsley, A.P. and Engel, A. (2000). Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy. *EMBO. J*. **19,** 2229–2236.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F. and Tsernoglou, D. (1994). Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367,** 292–295.

Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P. and Saier, M.H., Jr. (2003). Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology* **149,** 3051–3072.

Pearson, T.W., Saya, L.E., Howard, S.P. and Buckley, J.T. (1982). The use of aerolysin toxin as an aid for visualization of low numbers of African trypanosomes in whole blood. *Acta Trop*. **39,** 73–77.

Pugsley, A.P., Francetic, O., Driessen, A.J. and de Lorenzo, V. (2004). Getting out: protein traffic in prokaryotes. *Mol. Microbiol*. **52,** 3–11.

Quiocho, F.A. (1986). Carbohydrate-binding proteins: tertiary structures and protein-sugar interactions. *Annu. Rev. Biochem*. **55,** 287–315.

Ribardo, D.A., Kuhl, K.R., Boldogh, I., Peterson, J.W., Houston, C.W. and Chopra, A.K. (2002). Early cell signaling by the cytotoxic enterotoxin of Aeromonas hydrophila in macrophages. *Microb. Pathog*. **32,** 149–163.

Rossjohn, J., Buckley, J.T., Hazes, B., Murzin, A.G., Read, R.J. and Parker, M.W. (1997). Aerolysin and pertussis toxin share a common receptor-binding domain. *EMBO. J*. **16,** 3426–3434.

Rossjohn, J., Raja, S.M., Nelson, K.L., Feil, S.C., van, der, Goot, Fg, Parker, M.W. and Buckley, J.T. (1998). Movement of a loop in domain 3 of aerolysin is required for channel formation. *Biochemistry* **37,** 741–746.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol*. **279,** 485–499.

Sauvonnet, N. and Pugsley, A.P. (1996). Identification of two regions of Klebsiella oxytoca pullulanase that together are capable of promoting beta-lactamase secretion by the general secretory pathway. *Mol. Microbiol*. **22,** 1–7.

Sharma, P., Varma, R., Sarasij, R.C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M. and Mayor, S. (2004). Nanoscale organization of mul-

tiple GPI-anchored proteins in living cell membranes. *Cell* **116,** 577–589.

Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nature Rev. Mol. Cell Biol*. **1,** 31–39.

Sotgia, F., Razani, B., Bonuccelli, G., Schubert, W., Battista, M., Lee, H., Capozza, F., Schubert, A.L., Minetti, C., Buckley, J.T. and Lisanti, M.P. (2002). Intracellular retention of glycosylphosphatidyl inositol-linked proteins in caveolin-deficient cells. *Mol. Cell Biol*. **22,** 3905–3926.

Sousa, M.V., Richardson, M., Fontes, W. and Morhy, L. (1994). Homology between the seed cytolysin enterolobin and bacterial aerolysins. *J. Protein Chem*. **13,** 659–667.

Stathopoulos, C., Hendrixson, D.R., Thanassi, D.G., Hultgren, S.J., St Geme, J.W., 3rd and Curtiss, R., 3rd. (2000). Secretion of virulence determinants by the general secretory pathway in Gram-negative pathogens: an evolving story. *Microbes. Infect*. **2,** 1061–1072.

Thayumanavan, T., Vivekanandhan, G., Savithamani, K., Subashkumar, R. and Lakshmanaperumalsamy, P. (2003). Incidence of hemolysin-positive and drug-resistant Aeromonas hydrophila in freshly caught finfish and prawn collected from major commercial fishes of coastal South India. *FEMS Immunol. Med. Microbiol*. **36,** 41–45.

Tschödrich-Rotter, M., Kubitscheck, U., Ugochukwu, G., Buckley, J. and Peters, R. (1996). Optical single-channel analysis of the aerolysin pore in erythrocyte membranes. *Biophys. J*. **70,** 723–732.

Tsitrin, Y., Morton, C.J., El Bez, C., Paumard, P., Velluz, M.C., Adrian, M., Dubochet, J., Parker, M.W., Lanzavecchia, S. and Van Der Goot, F.G. (2002). Conversion of a transmembrane to a water-soluble protein complex by a single point mutation. *Nat. Struct. Biol*. **9,** 729–733.

Van den Burg, B., Vriend, G., Veltman, O.R., Venema, G. and Eijsink, V.G. (1998). Engineering an enzyme to resist boiling. *Proc. Natl. Acad. Sci. USA* **95,** 2056–2060.

van der Goot, F.G., Ausio, J., Wong, K.R., Pattus, F. and Buckley, J.T. (1993a). Dimerization stabilizes the pore-forming toxin aerolysin in solution. *The Journal of Biological Chemistry* **268,** 18272–18279.

van der Goot, F.G., Lakey, J.H., Pattus, F., Kay, C.M., Sorokine, O., Van Dorsselaer, A. and Buckley, T. (1992). Spectroscopic study of the activation and oligomerization of the channel-forming toxin aerolysin: Identification of the site of proteolytic activation. *Biochemistry* **31,** 8566–8570.

van der Goot, F.G., Wong, K.R., Pattus, F. and Buckley, J.T. (1993b). Oligomerization of the channel-forming toxin Aerolysin precedes its insertion into lipid bilayer. *Biochemistry* **32,** 2636–2642.

Walev, I., Bhakdi, S.C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl. Acad. Sci. USA*. **98,** 3185–3190.

Wang, G., Tyler, K.D., Munro, C.K. and Johnson, W.M. (1996). Characterization of cytotoxic, hemolytic Aeromonas caviae clinical isolates and their identification by determining presence of a unique hemolysin gene. *J. Clin. Microbiol*. **34,** 3203–3205.

Wang, J., Liu, H.L., Xu, C.M., Yang, Y., Lv, Z.J., Pang, H.Z. and Zhang, Z.N. (2003). Detection of erythrocytes deficient in glycosylphosphatidyl-inositol anchored membrane proteins in patients with paroxysmal nocturnal hemoglobinuria by the toxin HEC secreted by Aeromonas hydrophila J-1. *Hematology* **8,** 41–46.

Weiss, M.S., Wacker, T., Weckesser, J., Welte, W. and Schulz, G.E. (1990). The three-dimensional structure of porin from *Rhodobacter capsulatus* at 3 Å resolution. *FEBS Lett*. **267,** 268–272.

Wilmsen, H.U., Buckley, J.T. and Pattus, F. (1991). Site-directed mutagenesis at histidines of aerolysin from *Aeromonas hydrophila*: a lipid planar bilayer study. *Molecular Microbiology* **5,** 2745–2751.

Wilmsen, H.U., Leonard, K.R., Tichelaar, W., Buckley, J.T. and Pattus, F. (1992). The aerolysin membrane channel is formed by heptamerization of the monomer. *EMBO Journal* **11,** 2457–2463.

Wilmsen, H.U., Pattus, F. and Buckley, J.T. (1990). Aerolysin, a hemolysin from *Aeromonas hydrophila*, forms voltage-gated channels in planar bilayers. *J. Memb. Biol*. **115,** 71–81.

Wong, K. and Buckley, J. (1989). Proton motive force involved in protein transport across the outer membrane of *Aeromonas hydrophila*. *Science* **246,** 654–656.

CHAPTER

# 34

# *Clostridium septicum* pore-forming α-toxin

*Jody Melton and Rodney K. Tweten*

## INTRODUCTION

Descriptions of myonecrosis (gas gangrene) have been recorded in history since the Middle Ages. Bottini was able to clearly demonstrate the bacterial origin behind this disease in 1871; however, he was not able to isolate a causative organism (Bottini, 1871). The first of these organisms was identified in 1877 by Pasteur and Joubert and given the name *Vibrion septique* (Pasteur and Joubert, 1877). Today, this organism, now known as *Clostridium septicum*, is one of six histotoxic clostridia that are able to initiate gas gangrene (clostridial myonecrosis) in humans (MacLennan, 1962). Whereas most histotoxic clostridia primarily cause gangrene after contamination of a wound or injury with spores or vegetative cells, *C. septicum* is a major cause of non-traumatic gas gangrene and necrotizing enterocolitis in individuals who have colonic cancer, neutropenia, diabetes, leukemia, and various other predisposing conditions. Since no external trauma is required to initiate this disease, it is thought that the infection arises from an endogenous source, presumably the colon.

*C. septicum* elaborates various toxins and virulence factors that are likely to contribute to disease. The first of these was discovered and partially purified in 1944 by Bernheimer and given the name alpha toxin (AT) (Bernheimer, 1944). While the lethal and hemolytic activities of *C. septicum* were purified together by Bernheimer, the fact that AT was responsible for both of these activities was not confirmed until AT was purified to homogeneity nearly 50 years later by Ballard (Ballard *et al*., 1992). AT is secreted by *C. septicum* and exhibits the typical structure of a type II secreted protein with a 31-residue amino terminal signal peptide signal. Secreted AT exhibits a mass of 46,550 D, and it is produced as an inactive protoxin. As is described below, activation of AT requires the cleavage of an amino terminal propeptide of 45 amino acids. AT is the sole lethal factor secreted by *C. septicum* with a reported $LD_{50}$ of 10 μg $kg^{-1}$ in mice (Ballard *et al*., 1992). Upon injection with purified AT mice undergo shock and death, the same course seen in human patients with non-traumatic gas gangrene (Ballard *et al*., 1992). Thus, it appears that AT is the major cause of shock-like symptoms that occur during *C. septicum* non-traumatic gas gangrene.

Although many clostridial species produce toxins, AT is, so far, unique among the clostridia. AT was thought to have similarity to toxins from *C. chauvei* and *C. histolyicum*; however, neither of these organisms produce toxins that are cross-reactive with antibodies against AT (Ballard *et al*., 1992). AT is a pore-forming toxin that forms homo-oligomeric complexes on cell membranes consisting of five to seven monomers. This makes it similar in action to several non-clostridial bacterial toxins, including *Staphylococcus aureus* alpha hemolysin (Gouaux *et al*., 1997), the cytotoxin from *Pseudomonas aeruginosa* (Xiong *et al*., 1994), the protective antigen component of anthrax toxin (Benson *et al*., 1998), enterolobin from the Brazilian tree *Enterolobium contortisiliquum* (Fontes *et al*., 1997), and aerolysin from *Aeromonas hydrophila* (Howard and Buckley, 1985). The primary structure of AT (Imagawa *et al*., 1994; Ballard *et al*., 1995) displayed striking sequence similarity to aerolysin (72% similarity, 27% identity) (Ballard *et al*., 1995).

Based on the similarity of its primary structure and its functional characteristic with that of aerolysin, AT now belongs to the small and unusual class of aerolysin-like cytolytic toxins. The aerolysin-like family of cytolytic toxins is novel among all pore-forming toxin families in that it contains members from a Gram-positive bacterium (AT from *C. septicum*), a Gram-negative bacterium (aerolysin from *Aeromonas hydrophila*), and a eukaryotic organism (enterolobin from seeds of the Brazilian tree. *contortisiliquum*). Furthermore, the recent solution of the crystal structure of *C. perfringens* epsilon toxin suggests that it is the newest member of this family of toxins (Cole *et al*., 2004; Chapter 35, this volume). To date, this toxin family is the most phylogenetically diverse family of pore-forming toxins. The members of this family display a high degree of sequence similarity with aerolysin (27% with AT, 36% with enterolobin); however, there is no significant sequence homology between AT and enterolobin or epsilon toxin. The similarities in structure and function between AT and aerolysin have provided an important resource for the study of the structure and mechanism of this family of toxins.

## MECHANISM OF ACTION AND STRUCTURE OF α-TOXIN

AT follows a distinct, ordered path for pore formation and much about the sequential order of the steps involved in this path has been determined. AT is produced as a preprotoxin containing an N-terminal type II signal sequence and a C-terminal propeptide. Protoxin monomers are secreted from the bacterial cells and bind to glycophosphatidylinositol (GPI)-anchored eukaryotic cell surface receptors. Once bound, cleavage of a 5-kDa C-terminal covalently linked propeptide by a furin like cell surface protease produces activated monomers. Activated toxin monomers then oligomerize on the cell surface before inserting into the membrane and forming a pore (Sellman *et al*., 1997). The pore sizes generated by these oligomers in planar lipid membranes have been estimated at 1.5 nm for AT (Ballard *et al*., 1993) and 1.0 nm for aerolysin (van der Goot *et al*., 1993). For aerolysin, it has been shown that the oligomer contains 7 monomers (Wilmsen *et al*., 1992) (Moniatte *et al*., 1996). Due to the similarity in their pore size, it is believed that AT may also form a heptameric oligomer, but this remains to be proven.

The crystal structure of aerolysin was the first one determined for a pore-forming toxin (Parker *et al*., 1994) and has had a significant impact on elucidating structure-function relationships for this family of toxins. While a crystal structure for AT has not been solved, a molecular model for it has been generated, based on the crystal structure of aerolysin and the high degree of similarity (72%) between domains 2–4 of aerolysin and AT (Melton *et al*., 2004). As seen in Figure 34.1, aerolysin is a bi-lobal protein with a small lobe comprised of the first 83 amino acids, also called domain 1(D1), and a large lobe that consists of two domains (D2–3). AT lacks a region homologous to D1 of aerolysin, but is homologous to domains 2–3 of aerolysin, making it a single-lobed structure consisting of three domains (Figure 34.1). The structure of the large lobe of aerolysin and the model of AT are largely β-sheet in structure.

### Binding and cellular receptors

It has been well established that members of the aerolysin and AT bind GPI-anchored cell surface receptors. Cellular receptors for AT include the human folate receptor (hFR), the neuronal surface molecule contactin, and Thy-1 (CD90) (Gordon *et al*., 1999), as well as members of the SAG family of cell surface proteins of *Toxoplasma gondii* (Wichroski *et al*., 2002). Aerolysin receptors include Thy-1, contactin, the

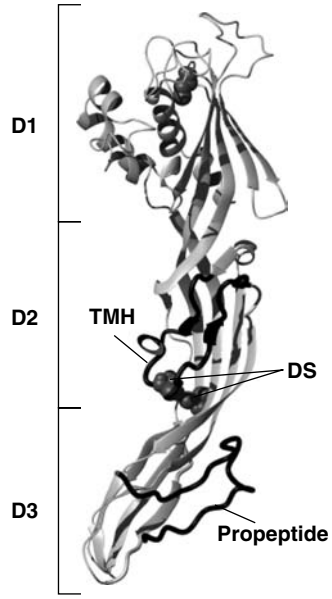



**FIGURE 34.1** Aerolysin-based structural model of α-toxin. Shown is a ribbon representation of α-toxin based on the aerolysin crystal structure (Parker *et al*., 1994). TMH: transmembrane β-hairpin (Melton *et al*., 2004), DS: engineered disulfide described by Melton *et al*. (Melton *et al*., 2004). The AT model was generated using Swiss model (Guex and Peitsch, 1997), and the ribbon structure was drawn using MolMol (Koradi *et al*., 1996).

47–kDa erythrocyte aerolysin receptor, and the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (MacKenzie *et al*., 1999). While aerolysin also binds contactin and Thy-1, it does not bind to hFR (Gordon *et al*., 1999). In a similar manner, AT cannot bind to the other identified aerolysin receptors. Thus, it appears that the two toxins bind to different sets of GPI-anchored proteins. Interestingly, the protein components of the AT- and aerolysin-binding GPI-anchored receptors lack sequence homology; however, all of the receptors identified for each toxin share a common property in that they are linked to the membrane through a GPI anchor. The GPI anchor appears to play a greater role in toxin binding than the protein to which it is attached.

The role of the GPI anchor in toxin binding has been of considerable interest in recent years. It has been established that the core of the GPI anchor itself acts as a binding determinant for aerolysin (Diep *et al*., 1998), and cell lines that are unable to synthesize GPI anchors have been shown to have a reduced or complete loss of sensitivity to aerolysin and AT, respectively (Gordon *et al*., 1999). However, the GPI anchor alone cannot function as a receptor for these toxins since not all GPI-anchored proteins are receptors (Diep *et al*., 1998; Abrami *et al*., 2002). It appears that the carbohydrate structure of the GPI anchor may be important, as the N-glycan core is necessary for aerolysin binding but not AT binding (Hong *et al*., 2002).

Previous work has identified residues in two of the four domains of aerolysin that are involved in receptor binding. Rossjohn *et al*. have shown that D1 of aerolysin contains a motif similar to the carbohydrate-binding domain of pertussis toxin, now named the aerolysin pertussis toxin (APT) domain (Rossjohn *et al*., 1997). As AT lacks a region homologous to D1 of aerolsyin, this observation may explain why the two toxins bind to different sets of GPI-anchored proteins. In fact, when the small lobe of aerolysin is fused to the amino terminus of AT, the hybrid toxin is able to bind receptors previously limited to aerolysin (Diep *et al*., 1999). Additional studies identified specific residues in domains 1 and 2 of aerolysin that participate in receptor binding. Using surface plasmon resonance, MacKenzie *et al*. found five site-specific mutations in D1 and four in D2 that had a significant effect on receptor binding (MacKenzie *et al*., 1999). Together, these data suggest a role for domains 1 and 2 of aerolysin in receptor binding. As AT lacks a region homologous to D1 of aerolysin, the results from aerolysin would suggest that at least a portion of the receptor-binding domain for AT would exist in D1 of AT (homologous to D2 of aerolysin).

## Activation and oligomerization of alpha toxin

Once protoxin is bound to the cell surface, the next step in the mechanism of action of these toxins is activation by a cell surface protease. Purified AT was a 48-kDa protein; however, most toxin preparations contained an additional 44-kDa protein that reacted to an antibody purified with the 48-kDa protein (Ballard *et al*., 1992). It was later determined that the 48-kDa protein represented an inactive protoxin requiring *in vitro* cleavage of a C-terminal propeptide by trypsin. It was subsequently shown that furin, and to a lesser extent proteases with a furin-like specificity, were necessary to activate AT on the surface of eukaryotic cells (Ballard *et al*., 1993; Gordon *et al*., 1997).

Proteolytic cleavage of the AT propeptide was necessary for its oligomerization into a pore-forming complex (Ballard *et al*., 1993). Therefore, the propeptide inhibited the interaction of membrane-bound monomers, and oligomerization only proceeded after AT had been proteolytically activated. Trypsin and furin cleave AT within a putative loop that is rich in basic amino acids. The furin consensus cleavage site is RXKR and is found within the AT motif of KKR-RGKR↓SVD. Cleavage occurs immediately after the furin recognition site of RGKR.

Sellman *et al*. (Sellman and Tweten, 1997) found that the propeptide of AT functions as an intramolecular chaperone by preventing the toxin from incorrect folding and premature aggregation in solution. When AT is activated in solution, the toxin forms solution aggregates that are not active on erythrocytes (Sellman and Tweten, 1997). If AT is activated in solution, it can form an oligomer, but this oligomer is incapable of interacting with and forming a pore on the membrane. They showed that in order for pore formation to occur, AT must follow an ordered pathway of assembly. First, monomers bind to the membrane via their GPI-anchored receptors and then are activated by protease. Only after binding and activation can the monomers oligomerize on the surface into functional pores. Therefore, the propeptide functions as an intramolecular chaperone by preventing improper association of the monomers until they reach the membrane where they are activated by furin.

Interestingly, the propeptide does not immediately dissociate from the toxin following proteolytic cleavage, but rather remains non-covalently associated with the monomer, and appears to be displaced during the oligomerization process (Sellman and Tweten,

1997). Thus, the covalent linkage of the propeptide with AT prevents its displacement until it is proteolytically activated. Furthermore, the addition of excess synthetic propeptide to activated AT monomers abrogates toxin activity by blocking oligomerization of activated monomers (Sellman and Tweten, 1997). Apparently, excess propeptide shifts the equilibrium onto the toxin and inhibits association of the monomers by occupying its position on the toxin, even in the absence of a covalent linkage. In the molecular model of AT the propeptide is located in domain 3 (Figure 34.1). The propeptide appears to protect an interfacial domain in domain 3 that is involved in monomer-monomer interaction.

The formation of a prepore complex has been demonstrated for several pore-forming toxins. The prepore complex is defined as a membrane-bound oligomer that has not formed the pore complex. Presumably the formation of the prepore complex organizes and coordinates the insertion of the membrane-spanning β-barrel of the pore-forming toxin. It has been shown that AT binds and oligomerizes on erythrocytes with nearly identical kinetics at both 4˚C and 37˚C; however, pore formation of the oligomer-containing membranes is completely abrogated at 4˚C (Sellman *et al*., 1997). The block in pore formation at 4˚C can be removed by a temperature shift to 37˚C, leading to the immediate formation of pores (Sellman *et al*., 1997). The fact that oligomers are present at 4˚C without pore formation supports the idea that AT oligomerizes into a prepore complex on the membrane surface prior to insertion.

## The transmembrane domain of AT and membrane insertion

Bacterial pore-forming toxins are unique proteins that exhibit an amphipathic nature in which they can exist in both soluble and membrane-associated states. Pore-forming toxins are often secreted from the bacteria into the surrounding milieu as water-soluble, hydrophilic monomers that bind to target cells, where they eventually protrude into the bilayer to reach their end-stage conformation as pore-forming homo-oligomers (Heuck *et al*., 2001). The fact that these proteins spontaneously insert into target membranes without the aid of complex machinery makes them distinctive from other end-stage, membrane-embedded proteins. Unlike colicins and other membrane-interacting proteins that contain long stretches of hydrophobic amino acids used to span the membrane, bacterial pore-forming toxins lack such hydrophobic regions in their sequence.

The crystal structure of the pore complex of the *Staphylococcus aureus* α-hemolysin by Song *et al*. (Song *et al*., 1996) provided considerable insight into the structure of a β-barrel pore. The pore was comprised of seven amphipathic β-hairpins that formed a 14-stranded β-barrel, a structure similar to that of the Gram-negative porins. The residue side chains comprising an amphipathic β-barrel have alternating hydrophobic/hydrophilic characters. Thus, once formed, one "side" of the barrel is capable of interacting with the hydrophobic membrane, while the other forms for interacting with the hydrophilic lumen of the pore. To date, the transmembrane structure of pore-forming toxins has often been determined to be an amphipathic β-strand. These include α-hemolysin from *Staphylococcus aureus* (Valeva *et al*., 1996), the protective antigen (PA) component of anthrax toxin (Benson *et al*., 1998), and perfringolysin O from *Clostridium perfringens* (Shepard *et al*., 1998; Shatursky *et al*., 1999). The size of the β-barrel formed by the various pore-forming toxins varies considerably; α-hemolysin and anthrax PA typically form heptameric oligomers, whereas the size of the PFO oligomer can vary from about 35–37 monomers (Czajkowsky *et al*., 2004), each of which contribute two transmembrane β-hairpins to the β-barrel structure.

Based on the crystal structure of aerolysin, it was hypothesized that D4 of aerolysin inserts three β-strands into the target membrane upon cleavage and removal of the propeptide (Parker *et al*., 1994). They suggested that removal of the propeptide following activation would expose a patch of hydrophobic residues in D4 of aerolysin capable of interacting with the hydrophobic core of the bilayer (Parker *et al*., 1994). A study on a point mutant of aerolysin that forms a water-soluble heptamer reiterated this idea, in which the authors hypothesized that the upper boundary of the transmembrane domain was distinguished by an aromatic belt in D4, similar to those found in porins (Tsitrin *et al*., 2002). However, no direct interaction of this domain with the membrane had been demonstrated. Other studies on aerolysin demonstrated that movement of a loop region in D3 of its structure (homologous to D2 of AT) is required for pore formation (Rossjohn *et al*., 1998). The authors were able to "lock" the loop region to the backbone of the molecule by mutating two residues (one on the loop region and one on the backbone) to cysteines and producing a disulfide bond. This form of aerolysin is completely inactive until a reducing reagent is added, at which time full activity is regained. In contrast to AT, aerolysin exists as a head-to-tail dimer in solution. As head-to-head oligomers are necessary for pore formation, the head-to-tail dimer must be dissociated prior to oligomerization of the prepore complex on the membrane. By locking this loop into place, the aerolysin

disulfide mutant failed to oligomerize, unless the disulfide was reduced. Thus, movement of the loop region in D3 of aerolysin may be required for head-to-tail dimer dissociation, pore formation, or both.

In contrast to aerolysin, AT does not appear to form a dimer in solution and so does not require dissociation of a dimer complex for membrane oligomerization. This aspect of AT simplified the search for the transmembrane domain of AT, and recently the membrane-spanning domain for AT was identified and determined to form an amphipathic β-barrel (Melton *et al*., 2004). The transmembrane domain of AT is comprised of the loop region in D2 of its molecular model, spanning from residues F200 to S235, and is homologous to the D3 loop of aerolysin discussed previously (Melton *et al*., 2004). This region was a candidate transmembrane domain based on the following observations. First, the primary structure of this region alternates between residues with hydrophobic and hydrophilic side chains, as would be expected for an amphipathic β-strand. Second, the manner in which the loop is extended from the backbone of the molecule in the molecular model appears to give it conformational freedom to move away from the backbone of the molecule and insert into the bilayer. This region was defined as the transmembrane domain by the use of various techniques. First, the construction of various sized deletion mutants that removed approximately 30% (Δ212–222), 60% (Δ208–226), or 90% (Δ204–231) of this region each demonstrated a complete loss of cytolytic activity, while retaining full receptor binding and oligomerization (Melton *et al*., 2004). As mentioned above, previous work on aerolysin in which an engineered disulfide bond was used to fasten the homologous loop region to the backbone of the molecule showed that movement of this region is required for pore formation. The analogous mutations were engineered into AT to determine if movement of the homologous region was required for pore formation. The disulfide-trapped mutant of AT bound to the receptor and oligomerized on membranes similar to wild-type AT; however, it was cytolytically inactive, unless the disulfide bond was reduced (Melton *et al*., 2004). These studies showed that movement of this loop in AT was required for the successful conversion of the prepore to the pore.

A direct interaction of this region with the membrane was demonstrated using the fluorescence-based approaches of Shepard *et al*. (Shepard *et al*., 1998) and Shatursky *et al*. (Shatursky *et al*., 1999), previously used for the identification and characterization of the transmembrane domains of PFO. These techniques take advantage of the environmentally sensitive properties of the sulfhydryl-specific fluorescent dye iodoacetamide-NBD (NBD). When NBD is attached to a residue that enters a hydrophobic environment such as the membrane, its fluorescent intensity will increase. On the other hand, when NBD is attached to a residue that remains in a hydrophilic environment, such as the lumen of the channel, its fluorescent intensity is quenched by water. When the residues of the loop region in D2 of AT (F200-S235) were individually mutated to cysteine and labeled with NBD, the fluorescence intensity changes detected upon insertion into membranes produced an alternating periodicity defining an amphipathic β-strand (Melton *et al*., 2004). The location of residues within the membrane was verified using a membrane-restricted collisional quencher of NBD, DOXYL-stearic acid. This lipid contains a nitroxide on the fatty acyl chain that is buried within the membrane. Nitroxides are efficient collisional quenchers of NBD; therefore, if the NBD resides within the membrane, its fluorescence will be quenched. When DOXYL-stearic acid was incorporated into the membranes, the NBD modified, cysteine-substituted residues that exhibited an increase in fluorescence intensity, when AT was membrane inserted, were quenched by the presence of the collisional quencher. Since this quencher could only quench NBD that was buried in the bilayer, these experiments confirmed that these residues formed a membrane-penetrating amphipathic β-hairpin (Melton *et al*., 2004).

## AT AND PATHOGENESIS

*C. septicum* is an infrequent cause of human infection, responsible for only 1.3% of all human clostridial infections (Kornbluth *et al*., 1989). While it is associated to the greatest extent with non-traumatic gas gangrene, it has also been associated with numerous other disease states, such as cerebritis (Roeltgen *et al*., 1980), meningitis (Dirks *et al*., 2000; Cheng *et al*., 1997), osteomyelitis (Neimkin and Jupiter, 1985; Shetty *et al*., 1998), necrotizing enterocolitis (Bignold and Harvey, 1979; Lev and Sweeney, 1993), necrotizing fasciitis (Schreuder and Chatoo, 1997), pericarditis (Brahan and Kahler, 1990), and as a superinfection of *E. coli* O157:H7-induced hemolytic uremic syndrome (Barnham and Weightman, 1998). No matter the disease course, infection with *C. septicum* is always accompanied by a predisposing condition, most notably malignancy. The association between *C. septicum* infection and malignancy has been detected in 81–85% of all known cases, and is occult in 35–37% of these cases at the time of

presentation (Alpern and Dowell, 1969; Kornbluth *et al*., 1989).

The most common form of malignancy associated with *C. septicum* infection is adenocarcinoma of the colon, found in up to 88% of all cases of *C. septicum* bacteremia and present in 84% of all cases with occult malignancies (Kornbluth *et al*., 1989). The relationship between *C. septicum* bacteremia and intestinal malignancy is so high that the bacteremia is often the first sign of an occult colonic carcinoma (Stevens *et al*., 1990).

The precise relationship between AT and *C. septicum* pathogenesis remains elusive. AT appears to be the sole lethal factor secreted by *C. septicum* (Ballard *et al*., 1992), but due to the difficulty of genetic manipulation of *C. septicum*, a gene knockout of AT is currently unavailable to assess its contribution to experimental myonecrosis. Several experiments using purified AT have produced convincing evidence that it is likely a major contributor to *C. septicum* myonecrosis. First, purified AT injected into mice induces rapid shock and death (Tweten and Brackett, unpublished results). AT-induced shock in these animal models is due to the sudden loss of fluid from the circulatory system, similar to the symptoms seen in the latter course of most human cases of *C. septicum* non-traumatic gas gangrene and necrotizing enterocolitis. In addition, immunization to AT provides protection to mice challenged with *C. septicum*. AT-immunized mice demonstrate a 20% mortality rate versus a 70% mortality rate in non-immunized animals when challenged with live *C. septicum* (Ballard *et al*., 1992). The ability of AT to exclusively mediate systemic effects has also been evidenced in a human case of *C. septicum*–associated meningeal vasculopathy, where complete lysis of vascular smooth muscle was observed in the absence of culturable organism or inflammation (Crowley *et al*., 1997). The immunogenicity of AT is also apparent in survivors of atraumatic gas gangrene, in whom AT has been shown to be the major immunodominant extracellular antigen (Johnson *et al*., 1994).

## CONCLUSION

A significant understanding of the structure-function relationships and cytolytic mechanism of AT, and that of the related aerolysin, has been gained in the last decade. It is likely that future investigations will focus on the contribution of AT to disease. It is apparent that several clostridial species can cause myonecrosis, yet they exhibit a varied array of lethal toxins that are not highly conserved among the histotoxic clostridia. For instance, *Clostridium septicum* produces the pore-forming AT, whereas *C. perfringens* produces an α-toxin that is a phospholipase C enzyme. Although each organism produces a different array of toxins, both cause virtually indistinguishable myonecrosis. Therefore, the central enigma to this disease is how lethal factors with very different mechanisms of actions contribute to its development.

Another interesting arena of investigation will be the study of the newest members of this family of toxins, enterolobin and *C. perfringens* epsilon toxin. Enterolobin is unusual since it is produced by a plant and is phylogenetically distant from the bacterial members of this family. Clearly, enterolobin is likely used in a very different way by the *E. contortisiliquum* tree than its bacterial counterparts. Little is understood about its mechanism or contribution to the life cycle of *E. contortisiliquum*. Whether epsilon toxin or enterolobin follow a similar mechanistic pathway to AT and aerolysin remains to be determined. The evolution of this family of toxins is also of interest, as it remains to be determined if the family members arose via divergent and/or convergent evolution. It is likely that the continued study of this family of toxins will reveal additional surprises and insights into how they function and contribute to the survival of their host species.

## ACKNOWLEDGMENTS

This work was funded by a grant from the National Institutes of Health (NIAID, AI37657).

## REFERENCES

Abrami, L., Velluz, M.C., Hong, Y., Ohishi, K., Mehlert, A., Ferguson, M., Kinoshita, T. and van der Goot, F.G. (2002). The glycan core of GPI-anchored proteins modulates aerolysin binding but is not sufficient: the polypeptide moiety is required for the toxin-receptor interaction. *FEBS Lett*. **512,** 249–254.

Alpern, R.J. and Dowell, V.J. (1969). *Clostridium septicum* infections and malignancy. *JAMA* **209,** 385–388.

Ballard, J., Bryant, A., Stevens, D. and Tweten, R.K. (1992). Purification and characterization of the lethal toxin (alpha toxin) of *Clostridium septicum*. *Infect. Immun*. **60,** 784–790.

Ballard, J., Crabtree, J., Roe, B.A. and Tweten, R.K. (1995). The primary structure of *Clostridium septicum* alpha toxin exhibits similarity with *Aeromonas hydrophila* aerolysin. *Infect. Immun*. **63,** 340–344.

Ballard, J., Sokolov, Y., Yuan, W.-L., Kagan, B.L. and Tweten, R.K. (1993). Activation and mechanism of *Clostridium septicum* alpha toxin. *Molec. Microbiol*. **10,** 627–634.

Barnham, M. and Weightman, N. (1998). *Clostridium septicum* infection and hemolytic uremic syndrome. *Emerg. Infect. Dis*. **4,** 321–324.

Benson, E.L., Huynh, P.D., Finkelstein, A. and Collier, R.J. (1998). Identification of residues lining the anthrax protective antigen channel. *Biochemistry* **37,** 3941–3948.

Bernheimer, A.W. (1944). Parallelism in the lethal and hemolytic activity of the toxin of *Clostridium septicum*. *J. Exp. Med*. **80,** 309–320.

Bignold, L.P. and Harvey, H.P. (1979). Necrotizing enterocolitis associated with invasion by *Clostridium septicum* complicating cyclic neutropaenia. *Aust. N. Z. J. Med*. **9,** 426–429.

Bottini, E. (1871). La gangrena traumatica invadente. Contribuzione sperimentali ed illutrazoni cliniche. *Giorn. Reale Accad. Med. Torino* **10,** 1121–1138.

Brahan, R. and Kahler, R.C. (1990). *Clostridium septicum* as a cause of pericarditis and mycotic aneurysm. *J. Clin. Microbiol*. **28,** 2377–2378.

Cheng, Y.T., Huang, C.T., Leu, H.S., Chen, J.S. and Kiu, M.C. (1997). Central nervous system infection due to *Clostridium septicum*: A case report and review of the literature. *Infection* **25,** 171–174.

Cole, A.R., Gibert, M., Popoff, M., Moss, D.S., Titball, R.W. and Basak, A.K. (2004). *Clostridium perfringens* epsilon-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat. Struct. Mol. Biol*. **11,** 797–798.

Crowley, R.S., Lembke, A. and Horoupian, D.S. (1997). Isolated meningeal vasculopathy associated with *Clostridium septicum* infection. *Neurology* **48,** 265–267.

Czajkowsky, D.M., Hotze, E.M., Shao, Z. and Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane β-hairpins to the membrane. *EMBO J*. **23,** 3206–3215.

Diep, D.B., Nelson, K.L., Lawrence, T.S., Sellman, B., Tweten, R.K. and Buckley, J.T. (1999). Expression and properties of an aerolysin-Clostridium septicum alpha toxin hybrid protein. *Mol. Microbiol*. **31,** 785–794.

Diep, D.B., Nelson, K.L., Raja, S.M., Pleshak, E.N. and Buckley, J.T. (1998). Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J. Biol. Chem*. **273,** 2355–2360.

Dirks, C., Horn, H., Christensen, L. and Pedersen, C. (2000). CNS infection with Clostridium septicum. *Scand. J. Infect. Dis*. **32,** 320–322.

Fontes, W., Sousa, M.V., Aragao, J.B. and Morhy, L. (1997). Determination of the amino acid sequence of the plant cytolysin enterolobin. *Arch. Biochem. Biophys*. **347,** 201–207.

Gordon, V.M., Benz, R., Fujii, K., Leppla, S.H. and Tweten, R.K. (1997). *Clostridium septicum* alpha toxin is proteolytically activated by furin. *Infect. Immun*. **65,** 4130–4134.

Gordon, V.M., Nelson, K.L., Buckley, J.T., Stevens, V.L., Tweten, R.K., Elwood, P.C. and Leppla, S.H. (1999). *Clostridium septicum* alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. *J. Biol. Chem*. **274,** 27274–27280.

Gouaux, E., Hobaugh, M. and Song, L. (1997). Alpha-hemolysin, gamma-hemolysin, and leukocidin from *Staphylococcus aureus*: distant in sequence, but similar in structure. *Protein. Sci*. **6,** 2631–2635.

Guex, N. and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18,** 2714–2723.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2001). Beta-barrel pore-forming toxins: intriguing dimorphic proteins. *Biochemistry* **40,** 9065–9073.

Hong, Y., Ohishi, K., Inoue, N., Kang, J.Y., Shime, H., Horiguchi, Y., van der Goot, F.G., Sugimoto, N. and Kinoshita, T. (2002). Requirement of N-glycan on GPI-anchored proteins for efficient binding of aerolysin but not Clostridium septicum alpha-toxin. *EMBO J*. **21,** 5047–5056.

Howard, S.P. and Buckley, J.T. (1985). Activation of the hole-forming toxin aerolysin by extracellular processing. *J. Bacteriol*. **163,** 336–340.

Imagawa, T., Dohi, Y. and Higashi, Y. (1994). Cloning nucleotide sequence and expression of a hemolysin gene of *Clostridium septicum*. *FEMS Microbiol. Lett*. **117,** 287–292.

Johnson, S., Driks, M.R., Tweten, R.K., Ballard, J., Stevens, D.L., Anderson, D.J. and Janoff, E.N. (1994). Clinical courses of seven survivors of *Clostridium septicum* infection and their immunological responses to α-toxin. *Clin. Infect. Dis*. **19,** 761–764.

Koradi, R., Billeter, M. and Wuthrich, K. (1996). MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph*. **14,** 51–55.

Kornbluth, A.A., Danzig, J.B. and Bernstein, L.H. (1989). *Clostridium septicum* infection and associated malignancy report of two cases and review of the literature. *Medicine* (Baltimore) **68,** 30–37.

Lev, R. and Sweeney, K.G. (1993). Neutropenic enterocolitis. Two unusual cases with review of the literature. *Arch. Path Lab. Med*. **117,** 524–527.

MacKenzie, C.R., Hirama, T. and Buckley, J.T. (1999). Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. *J. Biol. Chem*. **274,** 22604–22609.

MacLennan, J.D. (1962). The histotoxic clostridial infections of man. *Bact. Rev*. **26,** 177–276.

Melton, J.A., Parker, M.W., Rossjohn, J., Buckley, J.T. and Tweten, R.K. (2004). The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. *J. Biol. Chem*. **279,** 14315–14322.

Moniatte, M., van der Goot, F.G., Buckley, J.T., Pattus, F. and van Dorsselaer, A. (1996). Characterization of the heptameric pore-forming complex of the Aeromonas toxin aerolysin using MALDI-TOF mass spectrometry. *FEBS Lett*. **384,** 269–272.

Neimkin, R.J. and Jupiter, J.B. (1985). Metastatic nontraumatic *Clostridium septicum osteomyelitis. J. Hand. Surg. [Am]* **10,** 281–284.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F. and Tsernoglou, D. (1994). Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367,** 292–295.

Pasteur, L. and Joubert, P.A. (1877). Charbon et septicemie. *Bull. Acad. Med*. **6,** 781.

Roeltgen, D., Shugar, G. and Towfighi, J. (1980). Cerebritis due to Clostridium septicum. *Neurology* **30,** 1314–1316.

Rossjohn, J., Buckley, J.T., Hazes, B., Murzin, A.G., Read, R.J. and Parker, M.W. (1997). Aerolysin and pertussis toxin share a common receptor-binding domain. *EMBO J*. **16,** 3426–3434.

Rossjohn, J., Raja, S.M., Nelson, K.L., Feil, S.C., Van der Goot, F.G., Parker, M.W. and Buckley, J.T. (1998). Movement of a loop in domain 3 of aerolysin is required for channel formation. *Biochemistry* **37,** 741–746.

Schreuder, F. and Chatoo, M. (1997). Another cause of necrotizing fasciitis? *J. Infect*. **35,** 177–178.

Sellman, B.R., Kagan, B.L. and Tweten, R.K. (1997). Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. *Molec. Microbiol*. **23,** 551–558.

Sellman, B.R. and Tweten, R.K. (1997). The propeptide of Clostridium septicum alpha toxin functions as an intramolecular chaperone and is a potent inhibitor of alpha toxin-dependent cytolysis. *Molec. Microbiol*. **25,** 429–440.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: A novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Shepard, L.A., Heuck, A.P., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of a membrane-spanning domain of the thiol-act-

ivated pore-forming toxin *Clostridium perfringens* perfringolysin O: an α-helical to β-sheet transition identified by fluorescence spectroscopy. *Biochemistry* **37,** 14563–14574.

Shetty, A.K., Heinrich, S.D. and Steele, R.W. (1998). *Clostridium septicum* osteomyelitis: case report and review. *Ped. Infect. Dis. J.* **17,** 927–928.

Song, L.Z., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Stevens, D.L., Musher, D.M., Watson, D.A., Eddy, H., Hamill, R.J., Gyorkey, F., Rosen, H. and Mader, J. (1990). Spontaneous, nontraumatic gangrene due to *Clostridium septicum*. *Rev. Infect. Dis.* **12,** 286–296.

Tsitrin, Y., Morton, C.J., el-Bez, C., Paumard, P., Velluz, M.C., Adrian, M., Dubochet, J., Parker, M.W., Lanzavecchia, S. and van der Goot, F.G. (2002). Conversion of a transmembrane to a water-soluble protein complex by a single point mutation. *Nat. Struct. Biol.* **9,** 729–733.

Valeva, A., Weisser, A., Walker, B., Kehoe, M., Bayley, H., Bhakdi, S. and Palmer, M. (1996). Molecular architecture of a toxin pore: a 15-residue sequence lines the transmembrane channel of staphylococcal alpha-toxin. *EMBO J.* **15,** 1857–1864.

van der Goot, F.G., Pattus, F., Wong, K.R. and Buckley, J.T. (1993). Oligomerization of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. *Biochemistry* **21,** 2636–2642.

Wichroski, M.J., Melton, J.A., Donahue, C.G., Tweten, R.K. and Ward, G.E. (2002). *Clostridium septicum* alpha-toxin is active against the parasitic protozoan toxoplasma gondii and targets members of the sag family of glycosylphosphatidylinositol-anchored surface proteins. *Infect. Immun.* **70,** 4353–4361.

Wilmsen, H.U., Leonard, K.R., Tichelaar, W., Buckley, J.T. and Pattus, F. (1992). The aerolysin membrane channel is formed by heptamerization of the monomer. *EMBO J.* **11,** 2457–2463.

Xiong, G., Struckmeier, M. and Lutz, F. (1994). Pore-forming *Pseudomonas aeruginosa* cytotoxin. *Toxicology* **87,** 69–83.

CHAPTER

# 35

# *Clostridium perfringens* ε-toxin

*Ajit K. Basak, M. Popoff, R. W. Titball, and Ambrose Cole*

## INTRODUCTION

The genus *Clostridium* is a diverse and heterogeneous group of Gram-positive, anaerobic spore-forming bacteria. They are widely distributed in soil, sewage, and water. In addition, some species are present as part of the normal flora in the gastrointestinal tract of humans and animals. To date, over 100 different species of the *Clostridium* genus have been characterized and classified into different clusters on the basis of their 16s rDNA sequences. *Clostridium perfringens* is one member of this genus and is the causative agent of a wide variety of diseases, including gas gangrene, food poisoning, and necrotic enteritis in humans. The bacterium also causes a range of severe gastrointestinal and enterotoxemic diseases in domesticated livestock. The etiology of diseases caused by *C. perfringens* can be ascribed mainly to the production of various extracellular toxins, which are classified as either major or minor toxins. Based on the production of different major toxins (α-, β-, ε-, and ι-toxin), *C. perfringens* has been classified into five different groups (A, B, C, D, and F) (Table 35.1). In addition to the major toxins, the bacterium is also capable of producing a range of minor toxins such as θ-, λ-, ι-, μ-, κ-, and β2-toxin, as well as enzymes such as neuraminidase and urease (Laetitia *et al*., 1999; Rood, 1998). Not all these toxins have a proven role in virulence, and the recent genome sequence of *C. perfringens* type A has identified the presence of many more putative virulence genes whose functions are not yet known (Shimizu *et al*., 2002).

Recent evidence suggests that some of the toxins produced by this microorganism could be exploited as biological weapons. The activities of the United Nations Special Commission (UNSCOM, available at the Center for Non-proliferation Studies, Monterey Institute of International Studies, (http://www.cns.miis.edu/iiop/cnsdata) clearly identified *C. perfringens* as one of the microorganisms used to develop biological weapons by various aggressor states (Bowmann, 1998; Cordesman, 1998; Starr, 1998). The α-, β-, and ε-toxins and enterotoxin have all been suggested as candidate biological weapons in the past, but the ε-toxin is the most potent of this group with a mouse lethal dose ($LD_{50}$) of only 100ng/kg of body weight (Gill, 1982). This toxin is included in the Centers for Disease Control list of selected agents that might be used as biological weapons (Atlas, 1998).

In spite of research on ε-toxin over the past 50 years, its mode of action is still not clear. However, during the past decade some major advances have been made in understanding the molecular basis of toxicity. The aim of this article is to review these advances and summarize our knowledge of ε-toxin.

## ε-TOXIN

Of the five toxinotypes (A–E), only type B and D strains of *C. perfringens* produce ε-toxin (Brooks *et al*., 1957), and these strains are isolated mainly from the gut of diseased sheep and lambs, though occasionally from goats, cattle, and rarely from humans (Smith and Williams, 1984).

The ε-toxin, which has a predicted molecular weight of 32.5 kDa and 311 amino acids, is secreted as an inactive prototoxin (McDonel, 1986), which is activated to a mature lethal toxin by proteolytic cleavage (Bhown and Habeeb, 1977). Either the host or the bacterium

**TABLE 35.1** Diversity of *Clostridium perfringens* toxinotypes, genotypes, and the pathology associated with animals

| Toxinotype | Major toxin produced | Gene type and location | Associated pathology in domestic animals |
|---|---|---|---|
| A | α-toxin | *plc* / chromosome | Enterotoxemia in bovine and ovine, fowl necrotic enteritis, Myonecrosis, equine colitis, etc. |
| | α-toxin | *plc* / chromosome | Dysentery, chronic enteritis in lambs, entertoxemia in bovine and equine, bovine enteritis, etc. |
| B | β-toxin | *cpb1* / plasmid | |
| | ε-toxin | *etxB* / plasmid | |
| | α-toxin | *plc* / chromosome | Fowl necrotic enteritis, entertoxemia in ovine, bovine, equine, porcine, etc. |
| C | β-toxin | *cpb1, cpb2* / plasmid | |
| | α-toxin | *plc* / chromosome | Enterotoxemia in lambs and calves, bovine enterotoxemia. |
| D | ε-toxin | *etx*D / plasmid | |
| | α-toxin | *plc* / chromosome | Enterotoxemia in bovine and ovine, rabbit enteritis. |
| E | ι-toxin | *iap, ibp* / chromosome | |

provides the proteases, such as trypsin, chymotrypsin (Bhown and Habeeb, 1977; Hunter *et al*., 1992), or λ-protease (Jin *et al*., 1996; Minami *et al*., 1997) for cleavage of the prototoxin (Figure 35.1).

Maximum activation of the toxin occurs when a combination of trypsin and chymotrypsin is used, resulting in the loss of 13 N-terminal basic residues and 29 C-terminal residues, producing a mature toxin that is greater than 1,000-fold more toxic than the prototoxin, with an $LD_{50}$ of 70ng/kg. If activation occurs using pure trypsin, then cleavage at the C-terminus occurs seven residues later. This causes lower toxicity with an $LD_{50}$ of 320ng/kg. As mentioned, the toxin can also be activated by a protease secreted by *C. perfringens*, known as λ-toxin. This cuts the C-terminus at the same point as chymotrypsin, but leaves three extra residues at the N-terminus. The resulting activity is close to maximal with an $LD_{50}$ of 100ng/kg (Minami *et al*., 1997). The cleavage also causes a marked shift of pI from 8.02 for the prototoxin to 5.36 in the mature toxin, as well as a moiety with a pI of 5.74 thought to be partially activated toxin (Worthington and Mulders, 1977).

## GENETIC ARRANGEMENT

The ε-toxin encoding gene (*etx*) is located on a large plasmid (Canard *et al*., 1992) and appears to be associated with *IS1151* (Johnson, 1997), suggesting that it may be mobile. Both toxinotype genes (*etxB* and *etxD*) have been sequenced and only two differences have been found in the open reading frame (Figure 35.2). The first change, at position 762, does not result in an amino acid substitution. The second change, at position 962, results in a substitution from serine, in *etxB*, to tyrosine in *etxD* (Havard *et al*., 1992).

The upstream regions of the *etxB* and *etxD* genes are not identical with different putative −10 and −35 promoter regions (Figure 35.3). This suggests that expression of these genes may be regulated in different ways in type B and type D strains of *C. perfringens*. This possibility is supported by the observation that the strain from which the *etxD* gene was isolated (NCTC 8346) produced 10 times the amount of ε-toxin as the strain from which the *etxB* gene was isolated (NCTC 8533) (Havard *et al*., 1992). The prototoxin is expressed with a


**FIGURE 35.1** The points of cleavage of the ε-prototoxin to form the mature toxin by three proteases: trypsin, chymotrypsin, and λ-toxin.


**FIGURE 35.2** The genetic sequence of ε-Toxin, *etxB*. Underlined is the signal sequence for secretion and those letters in superscript refer to the sequence found in the *etxD* gene. In bold and italics are the regions cleaved for maximal activation (Hunter *et al*., 1992). Essential residues for lethal ativity are indicated by asterisks.

signal sequence of 32 amino acids that directs export of the toxin from the cell. When cloned into *Escherichia coli*, the signal sequence appears to direct export of the toxin across the cell membrane, resulting in accumulation of the toxin in the periplasmic space (Hunter *et al*., 1992).

## SEQUENCE ANALYSIS OF ε-TOXIN

The primary structure of ε-toxin bears no sequence similarity to any protein with a known structure in the current protein data bank (www.rcsb.org/pdb). However, the amino acid sequence of ε-toxin shows some homology to the *Bacillus sphaericus* mosquitocidal toxins Mtx2 and Mtx3, with 26% and 23% sequence identity, respectively. The *B. sphaericus* toxins and ε-toxin may have a similar mode of action, and both are activated by proteolytic cleavage (Liu *et al*., 1996; Thanabalu and Porter, 1996).

Studies on the chemical modification of ε-toxin have identified several essential amino acids, which include tryptophan, as well as some tyrosine and histidine residues (marked with an asterisk in Figure 35.2) that are essential for the activity of ε-toxin and its putative receptor binding to the membrane (Sakurai and Nagahama, 1985; Sakurai and Nagahama, 1987a; Sakurai and Nagahama, 1987b; Payne and Osten, 1997). Substitution of the two histidine residues, His119 or His162 (numbered from the prototoxin start), either with alanine or with serine, does not abolish

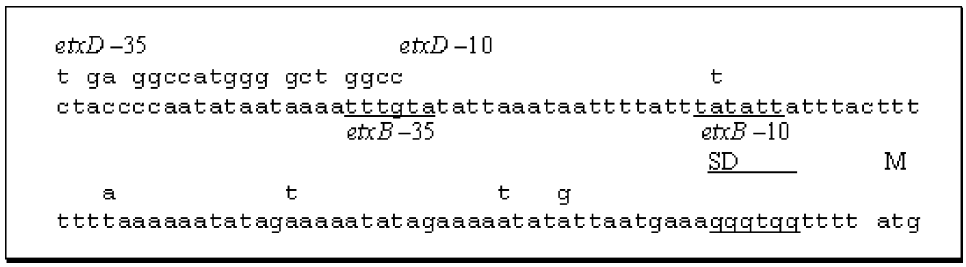



**FIGURE 35.3** The nucleotide sequence of the upstream promoter-binding region of both the *etxB* and *etxD* genes. Above the sequence is the prospective –35 and –10 regions for the *etxD* gene along with the substitutions. Below the sequence is the prospective *etxB* gene –35 and –10 regions. The Shine Dalgarno site (SD) is the same in both genes. The last three nucleotides code for the first methionine (M) of the signal sequence (Havard *et al*., 1992).

lethal activity; however, the change of His162 to alanine results in a significant decrease in lethal activity (Oyston *et al*., 1998).

## PATHOLOGY ASSOCIATED WITH INTOXICATION

Small amounts of toxin in the gut of animals are considered innocuous, but high concentrations lead to disease. For example, the toxin is responsible for a severe enteritis and an often fatal form of enterotoxemia in domestic livestock animals. *C. perfringens* type B is associated especially with dysentery in lambs, while *C. perfringens* type D is associated with enterotoxemia in sheep and lambs, along with a condition known as pulpy kidney or overeating disease (Bullen, 1970). These diseases are usually fatal and are characterized by a short period of time between the first appearance of symptoms and death. The mortality rate can be as high as 100%, and the diseases are of major economic significance, especially in industries based on intensive farming.

Pulpy kidney disease is closely correlated with changes in the microbial balance of the gut and often follows changes to the diet or treatment with antibiotics. Undigested food passed into the ileum creates a rich anaerobic environment in which *C. perfringens* can grow and multiply as high as $10^9$ cells per gram of ileum contents with each cell producing ε-toxin (Payne and Oyston, 1997). Crude vaccines exist for the prevention of disease associated with type B and D strains of *C. perfringens*. These vaccines are based on formaldehyde-treated cell filtrates or bacterial cells. The immunogenicity of these vaccines is variable (Percival *et al*., 1990), and the vaccines may not provide complete protection from disease. In this context, methods for the reliable production of ε-toxoid vaccines remain one of the challenges facing the veterinary vaccine industry.

The symptoms of disease, including neurological dysfunction and interstitial and pulmonary edema, can be reproduced by the administration of purified ε-toxin into goats and lambs (Uzal and Kelly, 1997; Uzal and Kelly, 1998), and generally appear within an hour. Many animals die peracutely, without premonitory signs (Niilo, 1993; Popoff, 1984). Peritoneal and pericardial effusions are common in naturally infected sheep (Jubb *et al*., 1993), and hyperglycemia and glycosuria are pathognomonic (Gardner, 1973; Niilo, 1993). Focal encephalomalacia, with bilaterally symmetrical brain lesions, is a chronic neurological manifestation of enterotoxemia (Buxton *et al*., 1981; Hartley, 1956). Other clinical signs of intoxication with ε-toxin include retraction of the head, opisthotonus (spasm of the body where the head and heels are bent backward and the body is bowed forward), convulsions, agonal struggling, hazard roaming, and head pressing, and they are often seen during the progression of this form of enterotoxemia (Songer, 1996).

A number of studies have shown that large doses of the toxin cause increased intestinal permeability, facilitating entry into the blood. The toxin then crosses the blood-brain barrier and accumulates in the brain as well as in the kidney, causing widespread osmotic alterations by disrupting vascular endothelia. These degenerative changes eventually cause serum proteins and red blood cells to leak out of the vasculature and cause massive edema (Finnie, 1984a) (Figure 35.4).

## NEUROTOXICITY

Pathological changes are apparent mainly within the brain, where necrotic brain lesions can be observed. Low doses of ε-toxin have been shown to damage the hippocampus, as well as the corpus striatum, cerebral cortex, vestibular area, corpus callosum, and corpus medullar cerebelli (Finnie, 1984b). In rats and mice, the

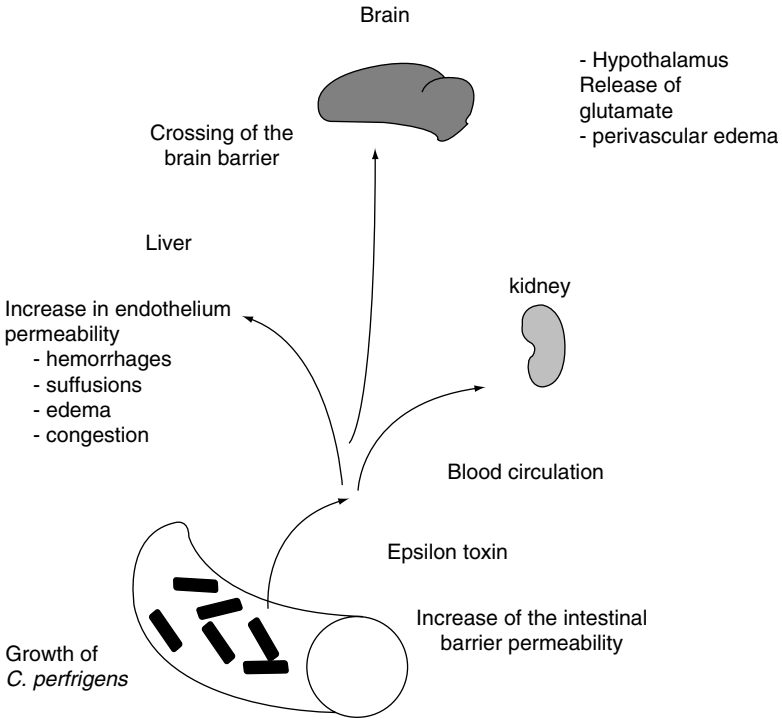



**FIGURE 35.4** Schematic representation of the ε-toxin mode of action.

hippocampal damage is associated with increased release of glutamate within specific regions (Miyamoto *et al*., 1998; Miyamoto *et al*., 2000). This increase in post-synaptic glutamate levels is apparently not the result of membrane damage because prior treatment of animals with glutamate release inhibitors reduces this effect and also gives some protection from intoxication. Cholinergic fibers in the same region are not affected, suggesting interaction with cells that use glutamate as a neurotransmitter. Focal-to-diffuse areas of degeneration and necrosis are common in affected animals (Buxton and Morgan, 1976), and bilateral macroscopic foci of encephalomalacia can occur (Buxton *et al*., 1981; Hartley, 1956; Finnie, 1984a; Finnie, 1984b). Clinical signs indicative of central nervous system (CNS) derangement, including incoordination and convulsions, are directly related to the severity of lesions (Griner *et al*., 1956). Vascular endothelial tight junctions degenerate (Buxton and Morgan, 1976), causing swelling and rupture of perivascular astrocyte processes (Finnie, 1984a). In mice, purified ε-toxin disrupts the permeability of the blood-brain barrier and allows the leakage of radiolabeled polyvinyl-pyrroliodone or human serum albumin into the brain, a discovery that led to the toxin first being labeled as a permease (Worthington and Mulders, 1975).

In sheep, intoxication with ε-toxin results in increased levels of cyclic adenosine 3', 5' mono-phosphate in the blood. This is thought to be a consequence of increased levels of ephedrine and norephidrine, which are, in turn, caused by the widespread edema (Worthington *et al*., 1979). Changes in these catecholamine levels have not been observed in mice (Nagahama and Sakurai, 1993). A significant drop in dopamine levels in brain tissue has been recorded in mice when large doses of toxin are administered. It has also been observed that drugs that inhibit the release of dopamine partially protect the mice from the lethal effects of ε-toxin (Nagahama and Sakurai, 1993).

Prior injection of mice with ε-prototoxin has also been shown to reduce the effects of the active toxin. This along with its clear preference for certain cell types indicates the presence of a specific receptor to which epsilon toxin binds. Activated ε-toxin has been shown to have a high affinity-binding site in the rat brain synaptosomal membrane (Nagahama and Sakurai, 1992) and has been suggested that the receptor is a sialoglycoprotein (Nagahama and Sakurai, 1992; Payne *et al*, 1997). The treatment of synaptosomal fractions of brain tissue with various enzymes has been used to try to ascertain what is important for ε-toxin binding. Pretreatment with a neuraminidase reduces binding, therefore suggesting that a sialoglycoprotein is important for the binding of ε-toxin (Nagahama and Sakurai, 1992).

## TOXICITY TOWARD CULTURED CELLS

A major step forward in the study of the mode of action of ε-toxin was the finding that Madin Darby Canine kidney (MDCK) cells were sensitive to ε-toxin (Knight *et al*., 1990; Payne *et al*., 1994). Subsequently, Lindsay and coworkers examined 17 different human cell lines along with MDCK cells and established that exposure of cells to 2μg/ml of ε-toxin reduced cell viability to 50% ($LC_{50}$) (Lindsay, 1996a). The only susceptible human cell line, Caucasian renal leiomyoblastoma (G-402) cells, was more resistant to intoxication with an $LC_{50}$ of 280 μg/ml.

*In vitro* exposure of MDCK cells with purified *C. perfringens* ε-toxin reveals evidence of cytoskeletal changes and irreversible damage to cells' plasma membrane (Donelli *et al*., 2003). MDCK cells become permeable to propidium iodide within two min of exposure to ε-toxin (Petit *et al*., 2001) and rapidly develop morphologic changes consistent with intoxication (Borrmann *et al*., 2001; Hambrook *et al*., 1995; Lindsay, 1996a; Petit *et al*., 1997; Petit *et al*., 2001). Cells subsequently swell, develop membrane blebs, and lyse (Borrmann *et al*., 2001; Petit *et al*., 1997; Petit *et al*., 2001) (Figure 35.5). However, there is no evidence of internalization of the toxin; the treatment of MDCK cells with sodium azide or agents that block endosome acidification (chloroquine, monensin, and bafilomycin A1) does not reduce toxicity, suggesting that endocytosis or other energy-dependent mechanisms are not required (Lindsay, 1996b; Petit *et al*., 1997).

The binding of ε-toxin to MDCK cells (and rat synaptosomal membranes) is associated with the formation of a stable high molecular weight complex (Nagahama *et al*.; 1998; Petit *et al*.; 1997). The formation of large complexes has also been observed with other pore-forming bacterial toxins, such as *Staphylococcus aureus* α-hemolysin (Song and Gouaux, 1998), *C. septicum* α-toxin (Melton *et al*., 2004), *Pseudomonas aeruginosa* cytotoxin (Ohnishi *et al*., 1994), and *Aeromonas hydrophilia* aerolysin (Wilmsen *et al*., 1992). Membrane fractions of neuronal cells exposed to $^{125}$I-labeled ε-toxin analyzed by SDS-PAGE and autoradiographed show the toxin migrating as two distinct bands. The first is a light band at 34 kDa, which approximately fits the predicted size of the monomer. The second is far heavier at 155 kDa and indicates the presence of some form of multimer. This 155-kDa band represents a highly stable, SDS-resistant complex of ε-toxin present in the membranes, allowing the efflux of $K^+$ (Petit *et al*., 1997).

Fully activated toxin is cleaved both at the N and C termini. Recombinant forms of the toxin, with these regions removed (Δ-C, Δ-N, and Δ-NΔ-C forms of the toxin), show differing abilities to form multimers. Constructs possessing the C-terminal sequence are never observed to form the large complexes, whereas those missing this sequence do (Miyata *et al*., 2001). The presence of two monomers, Δ-C and Δ-NΔ-C, with the ability to form a large complex have made it possible to ascertain the number of molecules present. Heterogeneous mixtures of the two forms produce autoradiographs with six intermediary bands present when using various molar ratios of the two constructs. This indicates that the complex formed is, in fact, a heptamer. This is inconsistent with the recorded size of the complex, as a heptamer of Δ-NΔ-C truncated toxin would have a mass of 198.25 kDa, whereas the recorded bands are between 155–180 kDa. But the heptameric form of the protein is SDS resistant and this may affect travel distance on a gel (Figure 35.6).

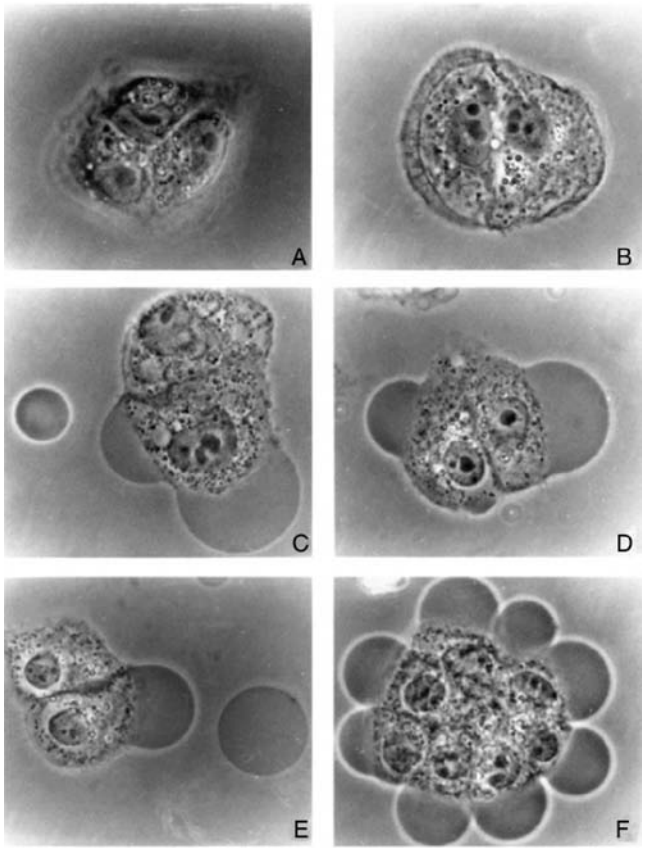



**FIGURE 35.5** Morphological changes of MDCK cells induced by ε-toxin. The cells were exposed to ε-toxin (50–375 ng/ml) for 60 minutes at 37°C in a 5% $CO_2$ incubator and were observed under phase contrast (magnification, X100). (A) Control cells; (B to F) cells exposed to ε-toxin at 50 ng/ml (B), 120 ng/ml (C and D), 210 ng/ml (E), and 375 ng/ml (F). Swelling is observed in panel B, and blebbing is observed in panels C to F. (Reproduced from *J. Bacteriol*, 1997, **179**, No. 20, 6480–6487, with permission from Prof. M. Popoff)

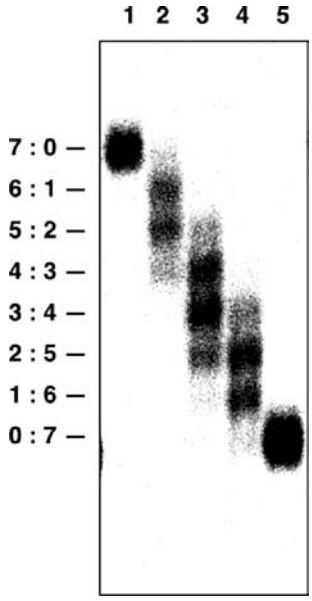



**FIGURE 35.6** Heptameric complex formation through copolymerization of [$^{32}$P]-Δ-C-PD and [$^{32}$P]-Δ-NΔ-C-PD. The proteins were mixed in different proportions: 1:0 (lane 1), 2:1 (lane 2), 1:1 (lane 3), 1:2 (lane 4), and 0:1 (lane 5). Each mixture (12 ng of protein) was incubated with synaptosomal membranes (2.2 μg of protein) in 12 μl of TBS. Samples were heated in SDS-sample buffer and separated on a 5% SDS-PAGE gel and autoradiographed. The predicted molar ratios of [$^{32}$P]-ΔC-PD to [$^{32}$P]-ΔNC-PD are listed on the left. (Reproduced from *J. Biol. Chem*. 2001, **276**, No. 17, 13778–13783, with the permission of Prof. A. Okabe.)

The possible pore-forming ability of ε-toxin has also been investigated via experiments using lipid bilayers. Activated ε-toxin added to bilayer membranes causes an increase in conductance across the membrane in a stepwise fashion after about two minutes. After about 30 minutes, the increase is of about three orders of magnitude (Petit *et al*., 2001). This stepwise increase indicates not only the presence of pores within the membrane after the addition of ε-toxin, but also that these pores are long lived with no association-dissociation equilibrium. Though various lipids have been used in these experiments, the toxin has not been shown to have any lipid preference (Petit *et al*., 2001). The size of these pores has also been investigated and shown to be at least 2 nm in diameter. The method, however, has limitations, as the polyethylene glycols (PEGs) used to size the pores have been shown to be slightly toxic to the MDCK cells.

Many pore-forming toxins have been shown to interact specifically with regions of the membrane known as detergent resistant micro-domains (DRMs). These regions within the membrane have a distinct liquid-ordered phase and preferentially contain distinct classes of proteins. Toxins such as aerolysin (Fivaz *et al*., 2001) and perfringolysin (Waheed *et al*., 2001), as well as their close relatives, all use DRMs as their main point of attack. These toxins share many similarities to ε-toxin with their pore-forming activity and mainly beta-sheet nature. Studies have therefore been carried out to ascertain ε-toxin's interaction with these regions. Both monomeric and heptameric toxin accumulates in this region, and depletion of cholesterol, a major constituent of DRMs, has an inhibitory effect on the toxin (Miyata *et al*., 2002). Additionally, the prototoxin is seen to bind mainly to DRMs. Although the prototoxin is unable to heptamerize, it is able to bind to the same receptor as that used by activated toxin, indicating that heptamerization is not a prerequisite for interactions with susceptible cells, but rather a result of binding. The putative receptor for ε-toxin is therefore thought to be present mainly in the DRMs, and all steps from binding to membrane insertion are thought to occur in these regions. The toxin is capable of forming channels in lipid bilayers easily without the presence of a receptor (Petit *et al*., 2001). This is also the case with other toxins, such as aerolysin, and has led to the proposition that as the receptors to which proteins bind are conserved to DRMs, they act in a manner so as to locally concentrate the toxins allowing heptamerization (Abrami and van Der Goot, 1999). However, ε-toxin is different from many pore-forming toxins in its specificity, because it only binds to very few cell types, unlike aerolysin, which binds to many GPI-linked proteins that are preferentially sequestered to DRMs.

## THE ε-TOXIN STRUCTURE

Recently, the 3D structure of ε-toxin was determined (Cole *et al*., 2004), although using the method of multiple anomalous diffraction as a model with enough similarity was not available in the current protein structure data bank (www.rcsb.org/pdb). The structure of ε-toxin is very elongated (100Å×20Å ×20Å) and is composed of mainly β-sheets (Figure 35.7a). The molecule can be divided into three domains with two strands (57–91 and 172–207) that travel the entire length of the molecule, covering a distance of approximately 130Å with only minor distortion occurring between domains. The first domain contains the largest α-helix (20–42), followed by a loop and then a short $3_{10}$ helix (46–49). Beyond this is the first strand of a three-stranded anti-parallel sheet (55–65, 197–206, and 214–220) upon which the large helix lies. In the region between domains one and two, there is a short $3_{10}$ helix (221–225) followed by a long loop (226–236). The region immediately after the long helix (42–52)

and part of the loop between domains (225–230) all exhibit some disorder as seen in the B-factors. The second domain is a β-sandwich containing a five-stranded sheet (62–82, 184–196, 237–248, 109–119, 150–160) and a two-stranded sheet (124–132, 141–146), both of which are anti-parallel. The final domain is also a β-sandwich motif, with one four-stranded (80–91, 172–185, 250–258, 262–267) and one three-stranded sheet (96–109, 161–170, 281–291). The final strand of the three-stranded sheet is the only parallel strand in the structure and forms a large part of the C-terminus that is removed on activation.

## COMPARISON OF ε-TOXIN AND AEROLYSIN

The overall fold of the ε-toxin structure shows some distinct similarity to aerolysin from the Gram-negative bacterium *Aeromonas hydrophila* (Parker *et al*., 1994). Despite the relatively poor sequence identity (~14%), both the structures show remarkably similar beta sheet arrangements (Figure 35.7a, b). In addition, ε-toxin shares many of the properties of aerolysin. Both toxins form heptameric pores (Moniatte *et al*., 1996) and both are secreted as prototoxins, which are activated by the removal of a C-terminal sequence by proteases.

One clear difference between the structures of ε-toxin and aerolysin is the presence of an additional domain in aerolysin (domain I in Figure 35.7b) (Parker *et al*., 1994). This domain has been postulated to be responsible for initial interaction with cells (MacKenzie *et al*., 1999). This initial binding can occur before activation, because furin, found in the eukaryotic cell membrane, is often the activating factor. However, furin is not preferentially located in detergent-resistant micro-domains (DRMs) where aerolysin

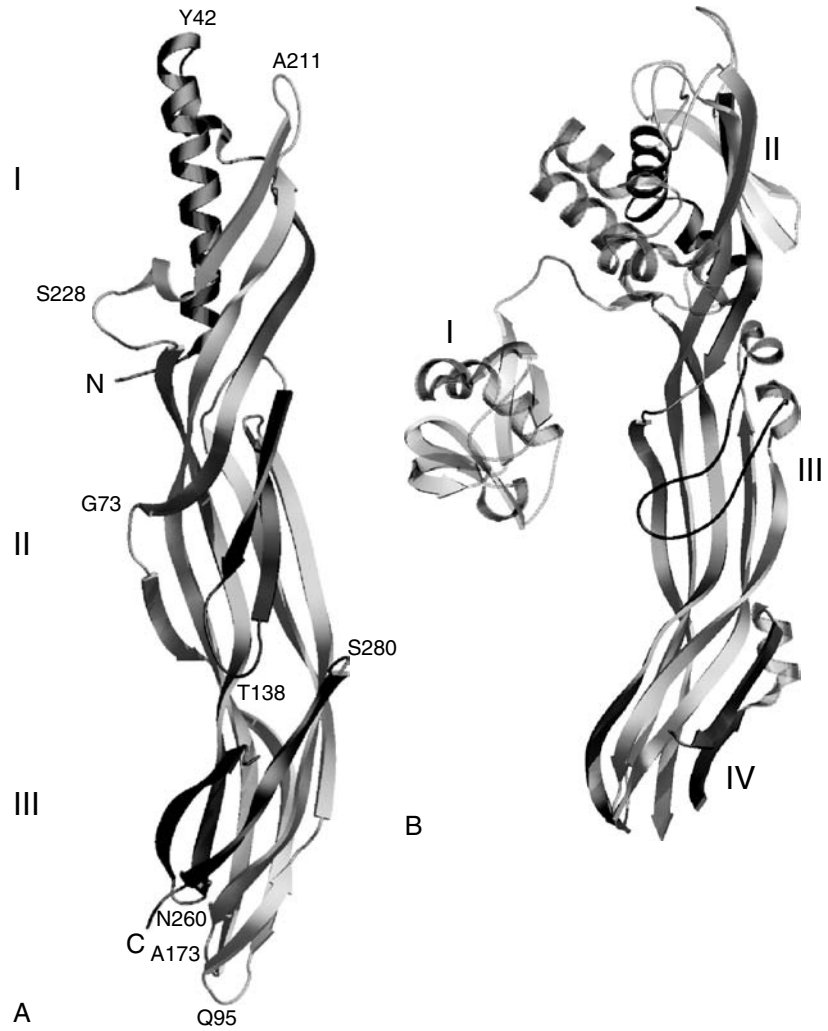



**FIGURE 35.7** Ribbon diagram of *C. perfringens* epsilon toxin **(a)** and *Aeromonas hydrophilia* aerolysin **(b)** colored from black at the N-terminus to medium-gray at the C-terminus. The additional domain in the aerolysin structure is colored in light-gray.

accumulates, therefore, some lateral movement of the toxin on the cell is required for activation.

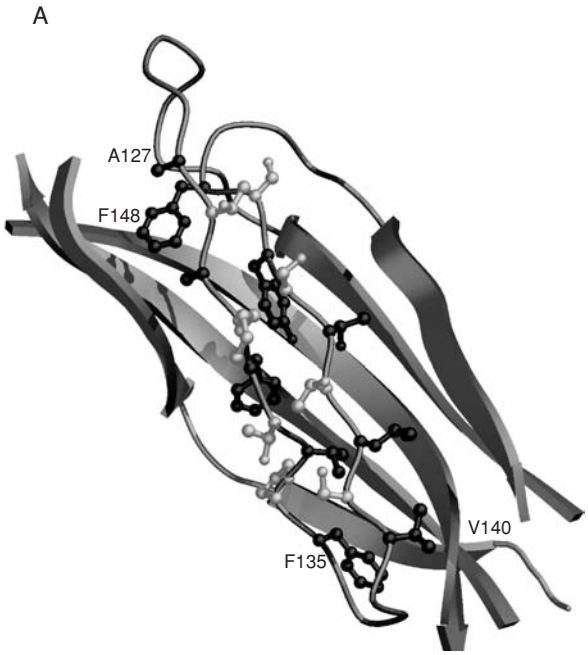
The first domain of ε-toxin shows some similarity to that of domain 2 of aerolysin (Figure 35.7a, b). In aerolysin this domain binds to the GPI anchors of proteins that are found in DRMs. It is possible that this domain performs a similar function in ε-toxin. If the roles of these domains are indeed similar, then the major reported differences in the target cell specificities of aerolysin and ε-toxin could be a function of the different structures and properties of these domains.

The second domain of ε-toxin exhibits obvious structural similarity to the third domain of aerolysin. On the basis of studies with *C. septicum* α-toxin, a homologous toxin to aerolysin, this domain in aerolysin has been proposed to be involved in membrane insertion (Melton *et al*., 2004). In both the ε-toxin and aerolysin structures, this domain is composed of a five-stranded sheet with a two-stranded sheet with an amphipathic sequence lying against it. An analogous region in *C. septicum* α-toxin to the two-stranded sheet was tested for insertion into a membrane, using sequential cysteine mutation, modified with a fluorescent probe sensitive to changes from an aqueous to a lipid environment. This technique showed that alternately these residues pointed into a lipid and then aqueous environment when bound to a membrane, indicating insertion of the two-stranded sheets in a similar manner to *S. aureus* α-toxin (Figure 35.8a, b).

The final domain of ε-toxin contains the C-terminus of the protein, cleavage of which is important for activation of the toxin. Interestingly, the carboxy termini of ε-toxin and aerolysin are located on opposite sides of the sheets in this domain. In the precursor forms of ε-toxin and aerolysin, the carboxy termini appear to block protein heptamerization. Both termini could therefore cover regions that form vital complementary surfaces required for oligomerization. The difference is that one C-terminus inhibits the interaction of the first with the second subunit of the heptamer, and the other inhibits the first with the seventh, given that the resulting structure would be a ring-shaped pore.

## CONCLUSION

Over the past several years, intense research on various biophysical, biochemical, and chemical properties, as well as the 3D structure of the toxin, has resulted in considerable progress in understanding the mode of




B

```
Epsilon toxin (139)  CKNTDTVTATTTHTVGTSIQATAKFTVPFNETG-VSLTTSYSFANTNTNTNSKEITHNVPSQD
Aerolysin (159)      YDTATNWSKTNTYGLSEKVTTKNKFKWPLVGETELSIEIAANQSWASQNGGSTTTSLSQSVR-
MT-X2 (130)          KSITDSTTTQTLNGFKTAFEASGKVGIPLVAEGQIKTTLEYNFSHTNSNTKSVTTTYTVPPQP
MT-X3 (121)          KSFSNTTTATTEHGFMFGTETSLATGIPFLAEGKITLKAEYNFSSSQANETSETVEYVAPSQS
α-toxin (188)        TSKTVSKTDNFKFGEKIGVKTSPKVGLEAIADSKVETSPEPNAEQGWSNTNSTTETKQESTTY
```

**FIGURE 35.8** (**a**) Residues predicted to point into the membrane are colored black whereas those pointing into the pore are colored gray. (**b**) Sequence alignment of the two stranded sheet region of epsilon toxin against the same region of aerolysin, the two mosquitociadal toxins and alpha toxin. The membrane and solvent exposed residues are shown in italic and bold respectively. Hydroxylated residues are shown underlined.

function of the *Clostridium perfringens* ε-toxin. The results of these studies will allow progress in understanding the molecular basis of toxicity and its role in various animal diseases. However, many key questions still remain unanswered, such as the mechanisms of interaction for ε-toxin to the cell membrane and the precise role of the amino acids for those membrane interactions, as well as the mechanism of oligomerization or the formation of the heptameric form. The structure of ε-toxin in its pore form is a major challenge for the future. The knowledge of structural reorganization that might take place upon activation will be an invaluable tool in elucidating the mechanics of its pore formation. This, in turn, will also help to design a candidate vaccine that can block the activity of ε-toxin.

Investigations need to be carried out about the significance of various residues throughout this toxin that have been previously identified to have some role in its biological activity. These residues include the single tryptophan (190), some tyrosine residues that have been shown to be important in binding to a receptor. To study the specific role of these residues in a possible binding site may lead to the discovery of prospective inhibitors.

Apart from the work on ε-toxin, 3D structures of other proteins with some homology, for example, mtx2 and mtx3 of *B. sphaericus*, may shed important information in the search for new methods in the control of malaria-causing mosquitos in the poor and developing world.

## REFERENCES


Abrami, L. and van Der Goot, F.G. (1999). Plasma membrane microdomains act as concentration platforms to facilitate intoxication by aerolysin. *J. Cell. Biol*. **147,** 175–184.

Atlas, R.M. (1998) Biological weapons pose challenge for microbiology community. *ASM News. Vol*. **64,** 383–389.

Bhown, A.S. and Habeeb, A.F. (1977). Structural studies on epsilon-prototoxin of *Clostridium perfringens* type D. Localization of the site of tryptic scission necessary for activation to epsilon toxin. *Biochem. Biophys. Res. Commun*. **78,** 889–896.

Borrmann, E., Schulze, F. and Diller, R. (2001). Development of *in vitro* methods for the potency testing of clostridial vaccines. *Altex*. **18,** 34–36.

Bowmann, E. (1998) Iraqi chemical and biological weapons capabilities. *Congressional Research Service*, Washington, DC. 1–5.

Brooks, M.E., Sterne, M. and Warrack, G.H. (1957). A reassessment of the criteria used for type differentiation of *Clostridia perfringens. J. Pathol. Bacteriol*. **74,** 185–195.

Bullen, J.J. (1970). Role of toxin in host-parasite relationship. In: *Microbial Toxins*. (eds. S. Ajl and S. Kadis), 1, pp. 233–276. Academic Press, New York.

Buxton, D., MacLeod, N.S.M. and Nicolson, T.B. (1981). Focal symmetrical encephalomalacia in young cattle, caused by *Clostridium welchii. Vet. Rec*. **108,** 459.

Buxton, D. and Morgan, K.T. (1976). Studies of lesions produced in the brains of colostrum-deprived lambs by *Clostridium welchii (Cl. perfringens)* type D toxin. *J. Comp. Pathol*. **86,** 435–447.

Canard, B., Saint-Joanis, B. and Cole, S.T. (1992). Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens. Mol. Microbiol*. **6,** 1421–1429.

Cole, A.R., Gibert, M., Popoff, M., Moss, D.S., Titball, R.W. and Basak, A.K. (2004). Clostridium perfringens epsilon toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat. Struct. Mol. Biol*. **11,** 797–798.

Cordesman. (1998) UNSCOM Main Achievements: *United Nations Special Commission* (UNSCOM). 67–91.

Donelli, G., Fiorentini, C., Matarresa, P., Falzano, L., Cardines, R., Mastrantonio, P., Payne, D. and Titball, R.W. (2003). Evidence for cytoskeletal changes secondary to plasma membrane functional alterations in the *in vitro* cell response to Clostridium perfringens epsilon toxin. *Microbiology and Infectious Disease*. **26,** 145–156.

Finnie, J.W. (1984a). Histopathological changes in the brain of mice given Clostridium perfringens type D epsilon toxin. *J. Comp. Pathol*. **94,** 363–370.

Finnie, J.W. (1984b). Ultrastructural changes in the brain of mice given Clostridium perfringens type D epsilon toxin. *J. Comp. Pathol*. **94,** 445–452.

Fivaz, M., Abrami, L., Tsitrin, Y. and van der Goot, F.G. (2001). Not as simple as just punching a hole. *Toxicon*. **39,** 1637–1645.

Gardner, D.E. (1973). Pathology of Clostridium welchii type D enterotoxaemia. I. Biochemical and hematological alterations in lambs. *J. Comp. Pathol*. **83,** 499–507.

Gill, D.M. (1982). Bacterial toxins: a table of lethal amounts. *Microb. Rev.* **46,** 86–94.

Griner, L.A., Aichelman, W.W. and Brown, G.D. (1956). Clostridium perfringens type D (epsilon) enterotoxemia in Brown Swiss dairy calves. *J. Am. Vet. Med. Assoc*. **129,** 375–376.

Hambrook, J.L., Lindsay, C.D. and Hughes, N. (1995). Morphological alterations in MDCK cells induced by exposure to Clostridium perfringens epsilon toxin. *Biochem. Soc. Trans*. **23,** 44S.

Hartley, W.J. (1956). A focal encephalomalacia of lambs. *N. Z. Vet. J*. **4,** 129–135.

Havard, H.L., Hunter, S.E. and Titball, R.W. (1992). Comparison of the nucleotide sequence and development of a PCR test for the epsilon toxin gene of Clostridium perfringens type B and type D. *FEMS Microbiol. Lett*. **76,** 77–81.

Hunter, S.E., Clarke, I.N., Kelly, D.C. and Titball, R.W. (1992). Cloning and nucleotide sequencing of the Clostridium perfringens epsilon toxin gene and its expression in Escherichia coli. *Infect. Immun*. **60,** 102–110.

Jin, F., Matsushita, O., Katayama, S., Jin, S., Matsushita, C., Minami, J. and Okabe, A. (1996). Purification, characterization, and primary structure of Clostridium perfringens lambda-toxin, a thermolysin-like metalloprotease. *Infect. Immun*. **64,** 230–237.

Johnson, E.A. (1997). Extrachromosomal virulence determinants in the clostridia. In: *The Clostridia: Molecular Biology and Pathogenesis* (eds. J.I. Rood, B. McClane, J.G. Songer and R.W. Titball), pp. 35–48. Academic Press, London.

Jubb, K.V.F., Kennedy, P.C. and Palmer, N. (1993). Pathology of domestic animals. Academic Press, London.

Knight, P.A., Queminet, J., Blanchard, J.H. and Tilleray, J.H. (1990). *In vitro* tests for the measurement of clostridial toxins, toxoids, and antisera. II. Titration of Clostridium perfringens toxins and antitoxins in cell culture. *Biologicals*. **18,** 263–270.

Laetitia, P., Gilbert, M. and Popoff, M.R. (1999). Clostridium perfringens: toxinotype and genotype. *Trends in Microbiol*. **7,** 104–110.

Lindsay, C.D. (1996a). Assessment of aspects of the toxicity of Clostridium perfringens epsilon toxin using the MDCK cell line. *Hum. Exp. Toxicol*. **15,** 904–908.

Lindsay, J.A. (1996b). Clostridium perfringens type A enterotoxin (CPE): more than just explosive diarrhea. *Crit. Rev. Microbiol*. **22,** 257–277.

Liu, J.W., Porter, A.G., Wee, B.Y. and Thanabalu, T. (1996). New gene from nine Bacillus sphaericus strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. *Appl. Environ. Microbiol*. **62,** 2174–2176.

MacKenzie, C.R., Hirama, T. and Buckley, J.T. (1999). Analysis of receptor binding by the channel-forming toxin aerolysin using surface plasmon resonance. *J. Biol. Chem*. **274,** 22604–22609.

McDonel, J.L. (1986). Toxins of Clostridium perfringens types A, B, C, D and E. In: *Pharmacology of Bacterial Toxins* (eds. F. Dorner and J. Drews), pp. 477–517. Pergamon Press, Oxford.

Melton, J.A., Parker, M.W., Rossjohn, J., Buckley, J.T. and Tweten, R.K. (2004). The identification and structure of the membrane-spanning domain of the Clostridium septicum alpha toxin. *J. Biol. Chem*. **279,** 14315–14322.

Minami, J., Katayama, S., Matsushita, O., Matsushita, C. and Okabe, A. (1997). Lambda-toxin of Clostridium perfringens activates the precursor of epsilon toxin by releasing its N- and C-terminal peptides. *Microbiol. Immunol*. **41,** 527–535.

Miyamoto, O., Minami, J., Toyoshima, T., Nakamura, T., Masada, T., Nagao, S., Negi, T., Itano, T. and Okabe, A. (1998). Neurotoxicity of Clostridium perfringens epsilon toxin for the rat hippocampus via the glutamatergic system. *Infect. Immun*. **66,** 2501–2508.

Miyamoto, O., Sumitani, K., Nakamura, T., Yamagami, S., Miyata, S., Itano, T., Negi, T. and Okabe, A. (2000). Clostridium perfringens epsilon toxin causes excessive release of glutamate in the mouse hippocampus. *FEMS Microbiol. Lett*. **189,** 109–113.

Miyata, S., Matsushita, O., Minami, J., Katayama, S., Shimamoto, S. and Okabe, A. (2001). Cleavage of a C-terminal peptide is essential for heptamerization of Clostridium perfringens epsilon toxin in the synaptosomal membrane. *J. Biol. Chem*. **276,** 13778–13783.

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S. and Okabe, A. (2002). Clostridium perfringens epsilon toxin forms a heptameric pore within the detergent-insoluble microdomains of MDCK cells and rat synaptosomes. *J. Biol. Chem*. **277,** 39463–39468.

Moniatte, M., van der Goot, F.G., Buckley, J.T., Pattus, F. and Van Dorselaer, A. (1996). Characterization of the heptameric pore-forming complex of the Aeromonas toxin Aerolysin using MALDI-TOFF mass spectrometry. *FEBS Lett*. **384,** 269–272.

Nagahama, M., Michiue, K., Mukai, M., Ochi, S. and Sakurai, J. (1998). Mechanism of membrane damage by Clostridium perfringens alpha-toxin. *Microbiol. Immunol*. **42,** 533–538.

Nagahama, M. and Sakurai, J. (1992). High-affinity binding of Clostridium perfringens epsilon toxin to rat brain. *Infect. Immun*. **60,** 1237–1240.

Nagahama, M. and Sakurai, J. (1993). Effect of drugs acting on the central nervous system on the lethality in mice of Clostridium perfringens epsilon toxin. *Toxicon*. **31,** 427–435.

Niilo, L. (1993). *Clostridium perfringens*. In: *Pathogenesis of Bacterial Infections in Animals* (eds. C.L. Gyles and C.O. Thoen), pp. 114–123. I.S.U Press.

Ohnishi, M., Hayashi, T., Tomita, T. and Terawaki, Y. (1994). Mechanism of the cytolytic action of Pseudomonas aeruginosa cytotoxin: oligomerization of the cytotoxin on target membranes. *FEBS Lett*. **356,** 357–360.

Oyston, P.C., Payne, D.W., Havard, H.L., Williamson, E.D. and Titball, R.W. (1998). Production of a non-toxic site-directed mutant of Clostridium perfringens epsilon toxin, which induces protective immunity in mice. *Microbiology* **144,** 333–341.

Parker, M.W., Buckley, J.T., Postma, J.P., Tucker, A.D., Leonard, K., Pattus, F. and Tsernoglou, D. (1994). Structure of the Aeromonas toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367,** 292–295.

Payne, D. and Oyston, P. (1997). The *Clostridium perfringens* ε-Toxin. In: *The Clostridia: Molecular Biology and Pathogenisis* (eds. J.I. Rood, B.A. McClane, J.G. Songer and R.W. Titball), Academic Press.

Payne, D.W., Williamson, E.D., Havard, H., Modi, N. and Brown, J. (1994). Evaluation of a new cytotoxicity assay for Clostridium perfringens type D epsilon toxin. *FEMS Microbiol. Lett*. **116,** 161–167.

Percival, D.A., Shuttleworth, A.D., Williamson, E.D. and Kelly, D.C. (1990). Anti-idiotypic antibody-induced protection against Clostridium perfringens type D. *Infect. Immun*. **58,** 2487–2492.

Petit, L., Gibert, M., Gillet, D., Laurent-Winter, C., Boquet, P. and Popoff, M.R. (1997). Clostridium perfringens epsilon toxin acts on MDCK cells by forming a large membrane complex. *J. Bacteriol*. **179,** 6480–6487.

Petit, L., Maier, E., Gibert, M., Popoff, M.R. and Benz, R. (2001). Clostridium perfringens epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J. Biol. Chem*. **276,** 15736–15740.

Popoff, M. (1984). Bacteriological examination in enterotoxaemia of sheep and lamb. *Vet. Rec*. **114,** 324.

Rood, J.I. (1998). Virulence genes of Clostridium perfringens. *Annu. Rev. Microbiol*. **52,** 333–360.

Sakurai, J. and Nagahama, M. (1985). Tryptophan content of Clostridium perfringens epsilon toxin. *Infect. Immun*. **47,** 260–263.

Sakurai, J. and Nagahama, M. (1987a). Histidine residues in Clostridium perfringens epsilon toxin. *FEMS Microbiol. Lett*. **41,** 317–319.

Sakurai, J. and Nagahama, M. (1987b). The inactivation of Clostridium perfringens epsilon toxin by treatment with tetranitromethane and N-acetylimidazole. *Toxicon*. **25,** 279–284.

Shimizu, T., Ohtani, K., Hirakawa, H., Ohshima, K., Yamashita, A., Shiba, T., Ogasawara, N., Hattori, M., Kuhara, S. and Hayashi, H. (2002). Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA*, **99,** No(2), 996–1001.

Smith, L.D.S. and Williams, B.L. (1984). *The Pathogenic Anaerobic Bacteria, 3rd ed*. (ed. C. Thomas), Springfield, IL.

Song, L. and Gouaux, E. (1998). Crystallization of the alpha-hemolysin heptamer solubilized in decyldimethyl- and decyldiethylphosphine oxide. *Acta Crystallogr D Biol Crystallogr*, **54** (Pt 2), 276–278.

Songer, J. G. (1996). Clostridial enteric diseases of domestic animals: a review. *Clin. Microbiol. Rev*. **9:** 216–234.

Starr, B. (1998) UNSCOM inspectors still doubt Iraq's arms claims. *Jane's Defence Weekly*, p. 18.

Thanabalu, T. and Porter, A.G. (1996). A Bacillus sphaericus gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. *Gene* **170,** 85–89.

Uzal, F.A. and Kelly, W.R. (1997). Effects of the intravenous administration of Clostridium perfringens type D epsilon toxin on young goats and lambs. *J. Comp. Pathol*. **116,** 63–71.

Uzal, F.A. and Kelly, W.R. (1998). Protection of goats against experimental enterotoxaemia by vaccination with Clostridium perfringens type D epsilon toxoid. *Vet. Rec*. **142,** 722–725.

Waheed, A.A., Shimada, Y., Heijnen, H.F., Nakamura, M., Inomata, M., Hayashi, M., Iwashita, S., Slot, J.W. and Ohno-Iwashita, Y. (2001). Selective binding of perfringolysin O derivative to

cholesterol-rich membrane microdomains (rafts). *Proc. Natl. Acad. Sci. USA* **98,** 4926–4931.

Wilmsen, H.U., Leonard, K.R., Tichelaar, W., Buckley, J.T. and Pattus, F. (1992). The aerolysin membrane channel is formed by heptamerization of the monomer. *EMBO. J.* **11,** 2457–2463.

Worthington, R.W., Bertschinger, H.J. and Mulders, M.S. (1979). Catecholamine and cyclic nucleotide response of sheep to the injection of Clostridium welchii type D epsilon toxin. *J. Med. Microbiol*. **12,** 497–501.

Worthington, R.W. and Mulders, M.S. (1975). Effect of Clostridium perfringens epsilon toxin on the blood brain barrier of mice. *Onderstepoort. J. Vet. Res*. **42,** 25–27.

Worthington, R.W. and Mulders, M.S. (1977). Physical changes in the epsilon prototoxin molecule of Clostridium perfringens during enzymatic activation. *Infect. Immun*. **18,** 549–551.

CHAPTER

# 36

# Repertoire and general features of the family of cholesterol-dependent cytolysins

*Joseph E. Alouf, Stephen J. Billington, and B. Helen Jost*

## INTRODUCTION

The cholesterol-dependent cytolysins (CDCs) (Shatursky *et al*., 1999; Tweten *et al*., 2001), also called cholesterol-binding cytolysins (Rossjohn *et al*. 1997; Alouf, 1999; Gilbert, 2000), constitute a group of 50- to 60-kDa single-chain, pore-forming bacterial protein toxins previously designated "sulfhydryl" (or "thiol-activated)" cytolysins. This group of structurally, antigenically, and functionally related cytolysins constitutes the largest family of bacterial protein toxins produced by Gram-positive bacteria. Moreover, the CDCs belong to a larger superfamily of structurally unrelated pore-forming toxins known as the beta-barrel pore-forming toxins (Heuck *et al*., 2001).

CDCs are lethal to animals and highly lytic towards eucaryotic cells, including erythrocytes. Their lytic and lethal properties are suppressed by sulfhydryl-group blocking agents and reversibly restored by thiols or other reducing agents. Another fundamental property of CDCs is the irreversible suppression of their biological activities by very low concentrations of cholesterol and other 3β-hydroxysterols. Membrane cholesterol is thought to be the toxin-binding site at the surface of target eukaryotic cells (see Morgan *et al*., 1996; Alouf, 1999, 2000, 2001; Billington *et al*., 2000; Palmer, 2001; Tweten *et al*., 2001).

While the CDCs have been named due to their dependence on cholesterol as part of the target membrane, other individual bacterial cytolysins, unrelated to the CDC family, also possess cholesterol-binding properties: *Burkholderia pseudomallei* (Ashdown and Koehler, 1990), *Vibrio cholerae* (Zitzer *et al*., 2003), and *Vibrio vulnificus* cytolysins (Kim and Kim, 2002; Chapter 44, this volume), the membrane-associated hemolysin of *Mycoplasma pulmonis* (Jarvill-Taylor and Minion, 1995), and the thiol-independent *E. coli* enterohemolysin (Figueiredo *et al*., 2003).

## REPERTOIRE OF THE CHOLESTEROL-DEPENDENT CYTOLYSINS

To date, the CDC family comprises 22 toxins produced by 24 Gram-positive bacterial species from seven genera (Table 36.1). The abbreviations of these toxins and of their structural genes are shown in Table 36.1.

Almost all CDC-producing species (except *Listeria seeligeri)* are pathogenic for humans and/or certain animals. However, *Bacillus thuringiensis, Brevibacillus* (formerly *Bacillus*) *laterosporus,* and *Paenibacillus* (formerly *Bacillus*) *alvei* are only pathogenic for certain insects, and very rarely in humans for the latter. Furthermore, among the CDC-producing species, only *Listeria monocytogenes* and *Listeria ivanovii* are intracellular pathogens that grow and release their toxins [(listeriolysin O (LLO) and ivanolysin O (ILO)] and various enzymes inside host phagocytic cells (Dramsi and Cossart, 2002). All CDCs are secreted into the extracel-

**TABLE 36.1** The family of structurally-related cholesterol-dependent cytolytic toxins

| Genus | Species | Toxin | Gene acronyms |
|---|---|---|---|
| *Streptococcus* | *S. pyogenes* | Streptolysin O (SLO) | *slo* |
| | *S. dysgalactiae* subsp. *equisimilis*[1] | Streptolysin O (SLO) | *Slo* |
| | *S. canis*[2] | Streptolysin O (SLO) | *slo* |
| | *S. pneumoniae* | Pneumolysin (PLY) | *ply* |
| | *S. suis* | Suilysin (SLY) | *sly* |
| | *S. intermedius* | Intermedilysin (ILY) | *ily* |
| *Bacillus* | *B. cereus* | Cereolysin O (CLO) | *clo* |
| | *B. thuringiensis* | Thuringiolysin O (TLO) | *tlo* |
| | *B. anthracis* | Anthrolysin O (ALO) | *alo* |
| *Brevibacillus* | *B. laterosporus* | Laterosporolysin (LSL) | *lsl* |
| *Paenibacillus* | *P. alvei* | Alveolysin (ALV) | *alv* |
| *Clostridium* | *C. tetani* | Tetanolysin (TLY) | *tly* |
| | *C. botulinum* | Botulinolysin (BLY) | *bly* |
| | *C. perfringens* | Perfringolysin O (PFO) | *pfo* |
| | *C. septicum* | Septicolysin O (SPL) | *spl* |
| | *C. histolyticum* | Histolyticolysin O (HTL) | *htl* |
| | *C. novyi A (oedematiens)* | Novyilysin (NVL) | *nvl* |
| | *C. chauvoei* | Chauveolysin (CVL) | *cvl* |
| | *C. bifermentans* | Bifermentolysin (BFL) | *bfl* |
| | *C. sordellii* | Sordellilysin (SDL) | *sdl* |
| *Listeria* | *L. monocytogenes* | Listeriolysin O (LLO) | *llo* |
| | *L. ivanovii* | Ivanolysin O (ILO) | *ilo* |
| | *L. seeligeri* | Seeligerolysin O (LSO) | *lso* |
| *Arcanobacterium* | *A. pyogenes* | Pyolysin (PLO) | *plo* |

[1]Group C streptococci
[2]Group G streptococci
Underlined: proteins purified to apparent homogeneity and sequenced genes

lular medium, except pneumolysin (PLY), which remains intracytoplasmic due to the lack of a signal peptide. This cytolysin is released outside *Streptococcus pneumoniae* cells upon bacterial autolysis (Johnson, 1977).

Streptolysin O (SLO), the most widely studied member of the CDCs, is considered as the eponymous archetype/prototype of this vast family of toxins (Bernheimer, 1976; Thelestam and Möllby, 1980; Pinkney *et al*., 1989; Bhakdi *et al*., 1996).

## HISTORICAL BACKGROUND AND IDENTIFICATION OF CHOLESTEROL-DEPENDENT CYTOLYSINS

CDCs have been progressively discovered over the past 106 years. Historically, they have been identified on the basis of their lytic effects on human and/or animal erythrocytes incubated with the supernatent fluids of bacterial cultures or by the formation of hemolytic zones around bacterial colonies on blood agar plates. Further investigations have showed that the toxins were also able to lyse and disrupt other eukaryotic cells (see "Cytolytic and Membrane-damaging Effects").

### The 1898–1960 period

In 1898, no less a person than Paul Ehrlich (1898) reported the presence of a hemolytic factor, tetanolysin (TLY), in *Clostridium tetani* culture fluids. This toxin was the first CDC discovered. Then Marmorek (1902) described the hemolytic effects of streptococcal cultures, which were probably due to a mixture of both streptolysin S (SLS) and the cholesterol-binding streptolysin O (SLO) produced by most *Streptococcus pyogenes* strains. These two cytolysins were differentiated later by Todd (1932, 1938) and Weld (1935). Pneumolysin (PLY) was revealed as early as 1901 by Tizzoni and Panichi (see Guillaumie, 1950), followed by Libman (1905) and Cole (1914). Since then, a number of investigations have been carried out on crude PLY preparations by Neill (see below), Cohen *et al*. (1940), and Halbert *et al*. (1946). The relationship of PLY to SLO and the inhibition of its hemolytic activity by cholesterol has been clearly established.

Perfringolysin O (PFO), previously known as θ-toxin, may have been detected first by Herter (1906–1907), but it is difficult to tell whether the author worked with this cytolysin or with *C. perfringens* α-toxin. PFO activities were clearly described later by Wuth (1923).

After these pioneering works, important achievements in the study of these toxins took place in the 1925–1945 period, particularly the systematic studies in the United States by Neill and his coworkers on PLY, PFO, TLY, and SLO (Neill, 1926 a, b, c; Neill and Mallory, 1926; Neill and Fleming, 1927). These authors developed the concept of the "oxygen-labile" nature of these toxins, which was extended to the other toxins of the group as they were discovered. This concept persisted in the literature until the end of the 1990s. These and other authors (Todd, 1934; Smythe and Harris, 1940; Herbert and Todd, 1941; Todd, 1941) observed that the hemolytic activities of crude or partially purified toxin preparations progressively disappeared on standing in air and less rapidly in the absence of oxygen. These activities were immediately restored by reducing agents, such as hydrosulfites or thiols, which are the most effective (Bernheimer and Avigad, 1970). The loss of activity was attributed (without direct chemical proof) to the oxidation of toxin molecules by $O_2$. However, this hypothesis was not supported by the finding that highly purified preparations of SLO, alveolysin (ALV), and PFO did not lose their lytic properties by flushing toxin solutions for several hours with air or $O_2$ (Alouf, 1980; Sato, 1986). The loss of activities in preparations observed by earlier investigators is probably due to a kinetically slow $O_2$-favored blockade of certain regions of toxin molecules (particularly the SH-group of the cysteinyl residue) by undefined molecules from culture media or bacterial metabolism. This, along with the discovery of members of this family that were truely not oxygen labile, led to the abandonment of the "old" term of oxygen-labile for the CDCs (Alouf, 1999; Billington *et al*., 2000).

Botulinolysin (BLY) produced by *Clostridium botulinum* was probably detected by Wheeler and Humphreys (1924) on the basis of the development of hemolytic zones around bacterial colonies on blood agar plates. This cytolysin was investigated in detail in the 1980s, as described in another section. Histicolysin O (ε-toxin) produced by *Clostridium histolyticum* was discovered by Mita (1934) and investigated by Howard (1953), who showed its "oxygen-labile" character, the inhibition of its hemolytic activity by cholesterol, as well as its antigenic relationship to SLO.

Septicolysin O (δ-toxin) produced by *Clostridium septicum* was detected by Eisenberg (1907) and later characterized by Moussa (1958). Sordellilysin produced by *Clostridium sordellii* was detected by Guillaumie and Kreger (1950). Oedematolysin (novyilysin) and bifermentolysin produced by *Clostridium oedematiens* and *Clostridium bifermentans* (*C. sordellii*), respectively, were discovered by Oakley *et al*. (1947) and Guillaumie (1950) and investigated later by Rutter and Collie (1969).

### The 1960–2003 period

The biochemical identification and purification of the *L. monocytogenes* CDC, listeriolysin O (LLO), was reported by Njoku-Obi *et al*. (1963) and Jenkins *et al*. (1964). However, earlier studies mentioned the production of a soluble hemolysin by this microorganism (Burn, 1934; Harvey and Faber, 1941). In 1970, Kingdon and Sword (1970) showed the inhibition of the hemolytic properties of the toxin by cholesterol. Thereafter, Geoffroy *et al*. (1987) purified LLO to apparent homogeneity and provided the unambiguous evidence that LLO is an SLO-related toxin belonging to the CDC family. The closely related ivanolysin O (ILO) and seeligeriolysin O (LSO) produced by *L. ivanovii* and *L. seeligeri*, respectively, were purified and characterized a few years later (Vazquez-Boland *et al*., 1989; Leimeister-Wächter and Chakraborty, 1989; Geoffroy *et al*., 1989).

In 1967, Bernheimer and Grushoff (1967a) reported the discovery of cereolysin O (CLO) produced by *Bacillus cereus*, which was shown to belong to the CDC family. Thuringiolysin O (TLO) was identified later (Pendelton *et al*., 1973) in the culture fluids of *Bacillus thuringiensis*, a species practically identical to *B. cereus*. Bernheimer and Grushoff (1967b) investigated several sporogenic bacilli and reported the detection of SLO-like "thiol-activated" cytolysins produced by *Bacillus* (now *Brevibacillus*) *laterosporus* and *Bacillus* (now *Paenibacillus*) *alvei*. Alveolysin (ALV), released by the latter, was purified and widely investigated in the early 1980s (Alouf *et al*., 1977; Geoffroy and Alouf, 1982, 1983), as described later in this chapter. New members of the CDC family were discovered and purified in the 1990s: suilysin (SLY) produced by *Streptococcus suis* (Jacobs *et al*., 1994), intermedilysin (ILY) produced by *Streptococcus intermedius* (Nagamune *et al*., 1996, 2000), pyolysin (PLO) produced by *Arcanobacterium pyogenes* (Funk *et. al*., 1996; Billington *et al*., 1997), and recently, anthrolysin O (ALO) produced by *Bacillus anthracis* (Shannon *et al*., 2003). The latter is very similar (98% identity) to CLO. New members of the CDC family may be discovered in the future.

## GENOMIC AND STRUCTURAL ASPECTS OF THE CHOLESTEROL-DEPENDENT CYTOLYSINS

The 16 structural genes of CDCs so far sequenced (Table 36.2) are all chromosomal. Interestingly, a number of these genes are located on genomic islands, suggesting that they were acquired by horizontal

**TABLE 36.2** CDCs with available gene sequences

| Toxins | References |
|---|---|
| Streptolysin O (*S. pyogenes*) | Kehoe *et al*. (1987, UK) |
| Streptolysin O (*S. dysgalactiae* subsp. *equisimilis*/*S. canis*) | Okumura *et al*. (1994, Japan) |
| Pneumolysin | Walker *et al*. (1987, UK) |
| Listeriolysin O | Mengaud *et al*. (1988, France), Domann & Chakraborty (1988, Germany) |
| Perfringolysin O | Tweten (1988, USA) |
| Alveolysin | Geoffroy *et al*. (1990, France) |
| Ivanolysin O | Haas *et al*. (1992, Germany) |
| Seeligerolysin O | Haas *et al*. (1992, Germany) |
| Cereolysin O | Yutsudo *et al*. (1994, Japan) |
| Intermedilysin | Nagamune *et al*. (1997, Japan) |
| Pyolysin | Billington *et al*. (1997, USA) |
| Suilysin | Segers *et al*. (1998, The Netherlands) |
| Anthrolysin O | Shannon *et al*. (2003, USA)[1] |
| Tetanolysin | GenBank accession NC_005957[1] |
| Thuringiolysin O | GenBank accession NC_004557[1] |

[1]The anthrolysin O, tetanolysin, and thuringiolysin O sequences were all deduced from bacterial genome sequences.

**TABLE 36.3** Molecular masses of mature CDCs derived from gene sequences

| Toxin | Mr (Da) | Number of amino acids |
|---|---|---|
| Streptolysin O | 60 151 | 538 |
| Seeligerolysin O | 56 371 | 505 |
| Ivanolysin O | 55 860 | 504 |
| Listeriolysin O | 55 842 | 504 |
| Tetanolysin | 55 533 | 495 |
| Intermedilysin | 55 244 | 502 |
| Pyolysin | 55 164 | 507 |
| Pneumolysin | 52 800 | 471 |
| Anthrolysin O | 52 704 | 478 |
| Thuringiolysin O | 52 691 | 478 |
| Cereolysin O | 52 500 | 473 |
| Perfringolysin O | 52 469 | 472 |
| Suilysin | 52 062 | 470 |
| Alveolysin | 51 766 | 469 |

transfer. The gene encoding LLO is located on the pathogenicity island LIPI-1 (Vazquez-Boland *et al*., 2001), while SLY and PLO genes are located on genomic islands, which may have been inherited through homologous recombination (Rudnick *et al*., 2003; Takamatsu *et al*., 2002). The amino acid sequence of the toxins deduced from the nucleotide sequences of the corresponding structural genes defines the 14 following toxins: SLO from *S. pyogenes*, *S. dysgalactiae* subsp. *equisimilis*, and *S. canis*, respectively, PLY, PFO, ALV, LLO, ILO, LSO, CLO, TLO, TLY, ILY, SLY, PLO, and ALO.

This family of toxins shows significant levels of amino acid similarity (about 40 to 70%), particularly in the carboxy-terminal portion of the proteins (Tweten *et al*., 2001; Shannon *et al*., 2003). Phylogenetically, CDCs tend to group based on closely related species, with PLO being the most divergent member of the family (Billington *et al*. 1997; Figure 36.1). However, SLO molecules are more closely related to the bacillus and clostridial CDCs than to pneumolysin or the other streptococcal CDCs (Figure 36.1). The molecular masses of the mature forms of the above-mentioned toxins range from about 60 to 51 kDa (Table 36.3). The longest amino acid sequence is that of SLO (60.2 kDa), while the smallest is that of ALV (51.8 kDa).

The largest region of sequence identity or homology in the proteins is an 11 amino acid region located near the C-terminus of the molecule, known as the undecapeptide or the Trp-rich region. This sequence is identical among 11 of the 14 sequenced CDCs (SLO, PLY, PFO, ALV, LLO, ILO, TLY, TLO, SLY, CLO, ALO) and is composed of the following sequence, ECTGLAWEWWR, containing the single cysteine residue of the toxin molecules and, conspicuously, three tryptophan residues (positions 7, 9, 10) (Figure 36.2).

The remaining three CDCs (PLO, ILY, and LSO) exhibited various substitutions in this "consensus" region, some of which are conservative and others which are not (Tweten, 2001). Very interestingly, PLO and ILY contained an alanine residue in position 2 instead of the cysteine residue, which for a long time

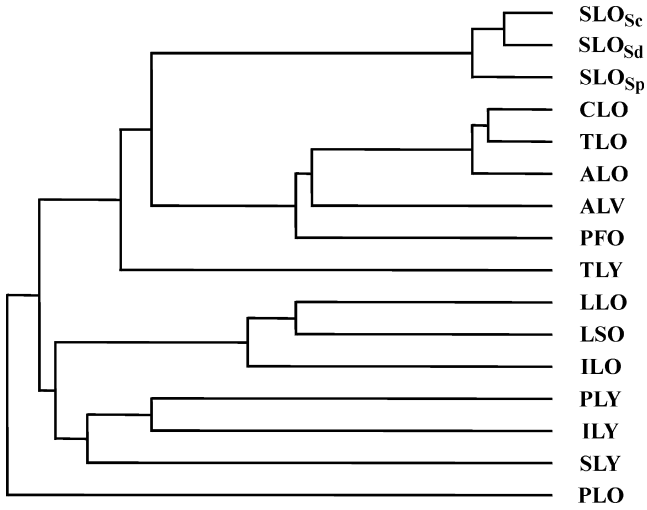



**FIGURE 36.1** Phylogenetic analysis of protein sequences showing the relationship between the cholesterol-dependent cytolysins. Cytolysins are indicated by their three-letter abbreviations, and SLO$_{Sp}$, SLO$_{Sd}$, and SLO$_{Sc}$ represent SLO molecules from *S. pyogenes*, *S. dysgalactiae* subsp. *Equisimilis*, and *S. canis*, respectively.

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SLO | 528 | I | M | A | R | E | C | T | G | L | A | W | E | - | W | W | R | K | V | I | D | 546 |
| PLY | 423 | V | K | I | R | E | C | T | G | L | A | W | E | - | W | W | R | T | V | Y | E | 441 |
| SLY | 451 | V | N | I | Q | E | C | T | G | L | A | W | E | - | W | W | R | T | V | Y | D | 469 |
| LLO | 479 | V | Y | A | K | E | C | T | G | L | A | W | E | - | W | W | R | T | V | I | D | 497 |
| LSO | 480 | I | Y | A | R | E | C | T | G | L | F | W | E | - | W | W | R | T | V | I | D | 498 |
| ILO | 478 | I | H | A | R | E | C | T | G | L | A | W | E | - | W | W | R | T | V | V | D | 496 |
| PFO | 454 | I | K | A | R | E | C | T | G | L | A | W | E | - | W | W | R | D | V | I | S | 472 |
| TLY | 480 | V | K | I | R | E | C | T | G | L | A | W | E | - | W | W | R | T | I | V | D | 498 |
| ALO | 466 | I | V | A | R | E | C | T | G | L | A | W | E | - | W | W | R | T | I | I | N | 484 |
| ALV | 456 | I | F | A | R | E | C | T | G | L | A | W | E | - | W | W | R | T | V | V | D | 474 |
| CLO | 467 | I | V | A | R | E | C | T | G | L | A | W | E | - | W | W | R | T | I | I | N | 485 |
| TLO | 467 | I | V | A | R | E | C | T | G | L | A | W | E | - | W | W | R | T | I | I | N | 485 |
| PLO | 486 | V | E | A | G | E | A | T | G | L | A | W | D | P | W | W | - | T | V | I | N | 505 |
| ILY | 481 | V | K | V | L | G | A | T | G | L | A | W | E | P | W | - | R | L | I | Y | S | 499 |

**FIGURE 36.2** Alignment of the amino acid sequences of cholesterol-dependent cytolysins within and around the conserved undecapeptide. Conserved amino acids within the motif are boxed in black. Amino acid numbers of precursor proteins are shown to the right and left of the sequences.

was thought strictly necessary for the biological activity of the CDC family. These two toxins were quite resistant to inactivation by sulfhydryl group blocking reagents and were fully biologically active (Billington *et al*., 1997; Nagamune *et al*., 1997). Moreover, PLO and ILY also have significant differences in the last four to five residues of the undecapeptide. In the latter, the glutamic acid residue (position 8) is replaced by glycine acid and the tryptophan residue (position 9) by proline. Therefore, ILY only contains two tryptophans in this region, while all of the other CDCs contain three tryptophans. PLO shows a conservative change of glutamic acid to aspartic acid (position 8), but this toxin also contains an insertion of a proline between positions 8 and 9, and lacks the terminal arginine residue (position 11) of the motif. In LSO, a single residue change in the undecapeptide occurs in which the alanine residue (position 5) is replaced by a phenylalanine. Interestingly, this toxin contains a second cysteine residue located outside the undecapeptide region nearer to the C-terminus.

## Structure-function relationship

The solving of the crystal structure of PFO monomer by Rossjohn *et al*. (1997) opened the door for a more systematic analysis of the roles of individual parts of the CDC molecule in biological activities. The 3D structure of PFO indicated an elongated protein of four domains, and while CDC primary amino acid sequences vary, the backbone of the 3D structure is fairly conserved. Hence, the four domain structure is probably representative of CDCs in general. Several models of pore formation have been proposed, with early models suggesting that insertion of a preformed pore would be energetically unfavorable, given the size of CDC pores (Palmer *et al*., 1998), but recent evidence favors the formation of a pre-pore complex prior to membrane insertion (Shepard *et al*., 2000). CDC structure-function relationships will be discussed in more detail in the following chapters. Here we will summarize the contribution of the four domains of the toxin to CDC biological function.

Based primarily on site-directed mutagenesis (Palmer *et al*., 1998; Abdel Ghani *et al*., 1999) and neutralization of epitopes with monoclonal antibodies (Darji *et al*., 1996), which abrogate oligomerization but not membrane binding, domain 1 has been implicated in subunit-subunit oligomerization. However, disulphide trapping and fluorescence experiments suggest that monomer-monomer interactions occur between β-strands on either side of domain 3 (Ramachandran *et al*., 2004), although residues involved in these interactions lie at the domain 1-domain 3 junctions.

Little information is available regarding the precise role of domain 2 in pore formation. However, this domain may act as a linkage domain, allowing significant movement of other domains relative to one another during membrane assembly.

The discovery that portions of domain 3 of the PFO molecule, which exist in the monomer as α-helices, are inserted into the host membrane (Shepard *et al*., 1998; Shatursky *et al*., 1999) revolutionized models of CDC pore formation in eukaryotic membranes. The results of Shatursky *et al*. (1999) confirmed the conversion of six domain 3 α-helices to two transmembrane β-hairpins (TMHs), which fully span the host membrane in the final pore, indicating significant structural alterations between the monomer CDC form and that of the membrane-inserted oligomer. Interestingly, two of the core β-strands that extend from the TMHs appear to be involved in oligomerization of the toxin on host membranes, with one of the interacting strands shielded in the monomer, presumably to prevent aberrant oligomerization in solution (Ramachandran *et al*., 2004).

Domain 4 was originally thought to be the portion of the molecule that inserted into the host membrane (Rossjohn *et al*., 1997). However, recent evidence suggests that domain 4 does not penetrate deeply into

the host membrane (Ramachandran *et al*., 2002), and that the TMHs of domain 3 actually form the faces of the inserted pore (Shatursky *et al*., 1999). Evidence from monoclonal antibody inhibition (de los Toyos *et al*., 1996) and toxin truncation studies (Tweten *et al*., 1991) indicates that domain 4 is involved in membrane cholesterol binding, but the fluorescence-based studies of Ramachandran *et al*. (2002) indicate that only the loops at the very tip of domain 4 move into the membrane. These authors suggest that domain 4 acts as a pivot for the reorientation of other portions of the molecule during membrane insertion. Domain 4 contains the characteristic CDC undecapeptide, which forms an extended loop at one end of the PFO 3D structure, and is included in the portion of domain 4 that accesses the membrane. The cysteine residue in this sequence is clearly responsible for the oxygen-labile nature of the majority of CDCs. Mutagenesis of the cysteine to alanine in PLY results in a toxin molecule that is fully active and unaffected by reducing agents (Saunders *et al*., 1989); CDCs that naturally lack this cysteine residue, PLO and ILY, are not oxygen labile (Billington *et al*., 1997; Nagamune *et al*., 1997); and introduction of the cysteine residue into PLO renders it thiol-active (Billington *et al*., 2002). A plethora of mutagenesis data indicates that the conserved CDC undecapeptide region is critical for the activities of most CDCs. In particular, mutagenesis of the three tryptophan residues results in significant effects on the cytolytic function of a number of CDCs (Boulnois *et al*., 1991; Michel *et al*., 1990; Sekino-Suzuki *et al*., 1996; Korchev *et al*., 1998; Billington *et al*., 2002). In addition, several studies have indicated that the tryptophan residues in this region move from a polar to a non-polar environment as the toxin moves into the host membrane (Nakamura *et al*., 1995; Heuck *et al*., 2000). The knowledge that domain 4 does not penetrate deeply into the host membrane (Ramachandran *et al*., 2002) and that the tryptophans of the undecapeptide sequence are close to the interface between the target membrane and the surrounding environment has led to the interpretation that the undecapeptide region may be involved in the sampling or initial tethering of the toxin to the host membrane.

## PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE TOXINS

Among the toxins listed in Table 36.1, only 16 (underlined) have been purified to apparent homogeneity, either as native or recombinant molecules, allowing subsequent characterization of their properties. The other toxins of the group have been practically disregarded so far since their discovery (see Guillaumie, 1950; Oakley *et al*., 1947; Moussa, 1958; Bernheimer and Grushoff, 1967b; Rutter and Collee, 1969; Hatheway, 1990) and will not be considered here. The purification procedures, characterization, physicochemical, molecular, and biological properties of SLO, PLY, PFO, CLO, ILY, and the listeriolysins are described in separate chapters of this volume. We will briefly describe here the following cytolysins: ALV, TLO, TLY, BLY, SLY, PLO, and ALO.

### Alveolysin (ALV)

This toxin is produced by *P. alvei,* as first reported by Bernheimer and Grushoff (1967b). No further characterization was performed until the systematic study of this toxin by Alouf and his coworkers. *P. alvei* is a soil organism that is often found in honeybee larvae suffering from European foulbrood. It is not considered pathogenic in vertebrates, although rare cases of human opportunistic infection have been reported (Reboli *et al*., 1989; Coudron *et al*., 1991). ALV was first purified by Alouf *et al*. (1977) by ammonium sulphate precipitation, gel filtration, isoelectric focusing, and ion-exchange chromatography. Toxin production was enhanced by about 10-fold ($2 \times 10^4$ HU/ml) in *P. alvei* shaken cultures in the presence of activated charcoal. ALV was then easily purified (specific activty $10^6$ HU/mg of protein) by thiol-disulphide interchange chromatography (Geoffroy and Alouf, 1983). The primary structure of the toxin was deduced from the nucleotide sequence of the gene (Geoffroy *et al*., 1990). The mature form comprised 469 amino acid residues (51766 Da) and contained a unique Cys residue in the consensus undecapeptide. The membrane-damaging activities of ALV were investigated on cultured human diploid lung fibroblasts (Thelestam *et al*., 1981). Toxin interaction with thiol group reagents, particularly tosyl lysine chloromethyl ketone (TLCK), prevented binding to erythrocytes and thereby hemolysis (Geoffroy and Alouf, 1982). The toxin was also used to permeabilize Raji cells and purified human peripheral blood lymphocytes. This procedure allowed investigation of the phosphoinositide pathway in these cells (Berthou *et al*., 1992).

### Thuringiolysin O (TLO)

This toxin produced by *B. thuringiensis* was first characterized by Pendleton *et al*. (1973), who obtained a partially purified preparation by precipitation of culture supernatants by ammonium sulphate followed

by isoelectric focusing. TLO was shown to focus into two distinct peaks of hemolytic activity at pI 6.0 and pI 6.5. The toxin was purified (specific activity $10^5$ HU/mg of protein) by ultrafiltration of the culture filtrates of strain H1–30 serotype 1, followed by chromatography on thiopropyl-Sepharose 6B columns (Rakotobé and Alouf, 1984). The toxin shared all the properties of the other "classical" antigenically related CDCs. The structural gene for TLO has been identified from the genome sequence of *B. thuringiensis*, and the deduced amino acid sequence is practically identical to CLO, with both toxins showing 98% amino acid identity with ALO (Shannon *et al*., 2003).

## Tetanolysin (TLY)

This toxin was first identified in 1898 in the culture filtrates of *Clostridium tetani* by Ehrlich (1898) (see also Neill, 1926c), who clearly differentiated it from the "true killing toxin" of *C. tetani* (tetanus neurotoxin) as established later by Hardegree (1965). Little information is available on TLY production by *C. tetani* strains. Lucain and Piffaretti (1977) reported a comparative study of TLY produced by nine different Tulloch serotypes of *C. tetani* as regards molecular weights, pIs, and electrophoretic mobilities. All TLY preparations were similar in these respects. The molecular weights measured by gel filtration were 47,000 ± 3,000 Da, and the pI values determined by isoelectric focusing in sucrose gradient were 6.54 for a major form, and 6.15 and 5.84 for minor forms, respectively. TLY purification from culture filtrates of Massachusetts C2 strain and Harvard A-47 strain was reported by Alving *et al*. (1979) and Mitsui *et al*. (1980), respectively. The procedure used by the former group involved ammonium sulphate precipitation, gel filtration, and chromatography. This procedure was modified by Rottem *et al*. (1990), who purified this toxin by sequential chromatography using Mono-Q and Sephadex G-100 columns. In both procedures, the purified toxin had a molecular weight of 45,000 Da and a specific activity of $10^6$ HU/mg. Two forms with pIs of 6.1 and 6.4 were found (Blumenthal and Habig, 1984). Recent deduction of the TLY amino acid sequence from the genome sequence of *C. tetani* confirms its similarity to other members of the CDC family.

## Botulinolysin (BLY)

This cytolysin produced by *C. botulinum* constitutes with eight other clostridial membrane-damaging toxins the largest subgroup of the CDC family (Table 36.1). A comparative study of toxin production in culture supernatants of six, three, and five strains from *C. botulinum* type C, D, and E, respectively, showed important variation in toxin production (140 to 3,400 HU/ml) (Haque *et al*., 1992). The high toxin-producing strain C-340 was used by these investigators for toxin purification. Culture supernatants were fractionated by ammonium sulphate precipitation, ion exchange, and gel filtration procedures and SP Toyopearl 650 M exchange column chromatography. The molecular weight of purified BLY was about 58 kDa. The 50% lethal dose in mice was 310 ng/ml and the 50% cytotoxic dose for Vero cells was 120 ng/ml.

Surprisingly, BLY was reported to contain four cysteine residues by amino acid analysis, in contrast to the single cysteine residues found in most other CDC molecules. BLY lysed the erythrocytes of many animal species, with higher lytic potency for rabbit, human, and guinea pig cells. The pharmacological activity of BLY on rat aortic ring and coronary vasoconstriction has been widely investigated (Sugimoto *et al*., 1995; 1997). Toxin damage on rabbit erythrocytes was studied by Sekiya *et al*. (1998) by electron microscopy, as described in another section.

## Suilysin (SLY)

SLY is produced by *S. suis*, a major cause of contagious porcine diseases, such as arthritis, septicemia, meningitis, pericarditis, endocarditis, polyserositis, and pneumonia. SLY was first purified by Jacobs *et al*. (1994) from culture supernatant by gel filtration and ammonium sulphate precipitation (specific activity 0.7 x $10^6$ HU/mg). Hemolytic activity was susceptible to oxidation and alkylation, inhibited by cholesterol, and increased by β-mercaptoethanol. SLY is hemolytic for erythrocytes from a number of animal species.

Molecular characterization of the *sly* gene confirmed that SLY is indeed a member of the CDC family, possessing the characteristic CDC undecapeptide (Segers *et al*., 1998) with a calculated molecular weight of 54,850 Da prior to signal sequence cleavage. Unlike other CDC-producing bacteria, not all *S. suis* strains possess the *sly* gene, and as such, not all strains produce SLY. The *sly* gene is more commonly present in virulent isolates and, interestingly, is present in a significant number of the predominant serotype 2 strains in Europe and Asia, but only in low numbers of North American serotype 2 isolates.

SLY is lytic for epithelial cells (Norton *et al*., 1999; Lalonde *et al*., 2000), which may facilitate access of *S. suis* to the bloodstream and brain microvascular endothelial cells (Charland *et al*., 2000; Vanier *et al*., 2004), which could lead to increased permeability of the blood-brain barrier, as well as phagocytic cells (Segura and Gottschalk, 2002).

However, consistent with the data that not all virulent *S. suis* strains contain *sly*, defined *sly* mutants, while avirulent in mice, are able to cause clinical signs in various pig models (Allen *et al*., 2001; Lun *et al*., 2003). Purified SLY protected mice against experimental *S. suis* infection, but afforded only partial protection in pigs (Jacobs *et al*., 1994; Jacobs *et al*., 1996).

## Pyolysin (PLO)

This CDC is produced by the commensal and opportunistic pathogen of livestock and wildlife, *A. pyogenes* (Ding and Lammler, 1996; Funk *et al*., 1996; Billington *et al*., 1997). First identified as a hemolytic toxin by Lovell (1937), it was not until its molecular characterization by Billington *et al*. (1997) that this toxin was recognized as a member of the CDC family, primarily because this was the first identified member of the family that was not subject to oxygen inactivation. PLO is an important virulence factor in *A. pyogenes* infections, as a *plo* knockout mutant is attenuated for virulence in a mouse model (Jost *et al*., 1999) and acts as an effective vaccine against experimental *A. pyogenes* infections (Billington *et al*., 1997; Jost *et al*., 1999; Jost *et al*., 2003). This toxin is lytic for erythrocytes, macrophages, and polymorphonuclear leucocytes from a variety of species (Ding and Lammler, 1996; Jost *et al*., 1999), as well as being dermonecrotic and lethal to laboratory animals by injection (Lovell, 1939; Lovell, 1944; Jost *et al*., 1999). Purification of recombinant PLO indicated a specific activity of $1.25 \times 10^5$HU/mg. While clearly a member of the CDC family of toxins, PLO shows some important differences. Phylogenetically, it is the most diverse member of the family (Figure 36.1; Billington *et al*., 1997) and exhibits 31 to 41% similarity with other CDCs. In addition, PLO, along with ILY, have C-terminal undecapeptide sequences, which diverge significantly from the consensus for this family (Figure 36.2; Billington *et al*., 1997; Nagamune, 1997). PLO lacks the conserved cysteine residue at position 2 of the undecapeptide, substituted with alanine, which is responsible for the thiol-activated nature of CDCs (Figure 36.2; Billington *et al*., 1997). This finding is consistent with the insensitivity of PLO activity to reducing agents. Mutagenesis of the alanine residue to cysteine conferred thiol activation on PLO, confirming the importance of the cysteine residue in this function (Billington *et al*., 2002). In addition, the PLO undecapeptide contains a conservative glutamic acid to aspartic acid substitution at position 7, the insertion of a proline residue between positions 7 and 8, and, finally, lacks the terminal arginine residue of the consensus motif. The divergent undecapeptide sequence of PLO appears to be required for its cytolytic activity, as mutations that bring the undecapeptide sequence closer to consensus, including a substitution with the consensus undecapeptide sequence, resulted in PLO molecules with low hemolytic activities (Billington *et al*., 2002).

## Anthrolysin O (ALO)

Databases of microbial genomes have allowed the identification of CDC homologues within the genome sequences of a number of bacterial species. One such homologue is ALO, the CDC of *B. anthracis*, which is closely related to CDCs produced by other *Bacillus* species, including TLY and CLO (Shannon *et al*., 2003). ALO is hemolytic against sheep and human red blood cells, although *B. anthracis* is not hemolytic on sheep blood agar plates. ALO is particularly active against human erythrocytes, with as few as 24 molecules of recombinant ALO per erythrocyte resulting in cell lysis. This toxin shares the properties of other CDCs, in that its activity is increased by reducing agents and inhibited by cholesterol.

# STEROL-BINDING PROPERTIES

The most characteristic biochemical property of CDCs is the specific and irreversible inhibition of their lytic, lethal cardiotoxic, and other biological properties by very low (nanomolar) amounts of cholesterol and certain structurally related sterols (Alouf, 1977, 1980; Cowell and Bernheimer, 1978; Smyth and Duncan, 1978). The inactivation of the other toxins by cholesterol and other sterols was subsequently demonstrated (Geoffroy and Alouf, 1983; Geoffroy *et al*., 1987; Ohno-Iwashita *et al*., 1988; Vazquez-Boland *et al*., 1989; and other authors as mentioned in this and other chapters).

## Structural requirements

The structural and stereospecific features necessary for inhibition of toxin were investigated in detail on quantitative bases for PLY, SLO, and PFO (see Watson and Kerr, 1974; Prigent and Alouf, 1976; and the abovementioned references). All inhibitory sterols possessed (i) an OH group in the β configuration on carbon-3 of ring A of the cyclopentanoperhydrophenanthrene nucleus, such as is found in cholesterol and kindred sterols; (ii) a lateral aliphatic side chain of suitable size (isooctyl chain in the case of cholesterol) attached to carbon-17 on the D ring; (iii) the presence of a methyl group at C-20; and (iv) an intact B ring. Neither the saturation state of the B ring nor the positions of the

double bonds or the stereochemical relationships of rings A and B were critical factors. The presence of an α-OH group on C-3 (epicholesterol), esterification of the β-OH group, or its substitution with a keto group or with a 3β-SH group (thiocholesterol) rendered the sterol inactive (Alouf and Geoffroy, 1979), presumably because such substitutions or orientations preclude correct presentation of the reactive 3β-OH group to the toxins.

## Characteristics of toxin-sterol interaction

Early experiments in liquid phase suggested the formation of complexes between toxin and sterol molecules (Smyth and Duncan, 1978; Cowell and Bernheimer, 1978). The first direct demonstration of the occurrence of these complexes, and thereafter, their separation, were provided by Alouf and his coworkers. The complexes were visualized by allowing SLO or ALV to diffuse from wells in sterol-containing agar gels (Prigent and Alouf, 1976; Alouf and Geoffroy, 1979; Geoffroy and Alouf, 1983). The complexes formed appeared as white, opaque halos (around toxin wells), constituted by insoluble (hydrophobic) precipitates of irreversibly bound toxin-sterol material stainable by protein dyes. Only inhibitory sterols showed such a pattern.

The separation of toxin-sterol complexes formed in liquid phase was reported by Johnson *et al*. (1980), who mixed pure PLY, SLO, or ALV preparations with [$^3$H]cholesterol solutions at concentrations in which this sterol was present in a soluble micellar form. Advantage was taken of the fact that when solutions of cholesterol in phosphate buffer are added to Sephadex or Sephacryl columns, the free cholesterol molecules stick to the gel and only toxin-sterol complexes elute with buffer. The separated complexes were hemolytically inactive and of high molecular weight. The amounts of bound sterol increased linearly with toxin concentration. The reaction was rapid and temperature independent, similar to the binding step of toxin interaction with target cells. The specificity of cholesterol binding was assessed by adding unlabeled inhibitory or non-inhibitory sterols to toxin before adding [$^3$H]cholesterol. Epicholesterol caused only a small decrease of binding, whereas 7-dehydrocholesterol inhibited radiolabeled binding to an extent equal to that observed with unlabeled cholesterol. Hemolytically inactive SLO obtained by treatment with oxidized dithiothreitol or by reaction with parahydroxymercuribenzoate showed no decrease in cholesterol-binding activity, whereas the ability of the toxins to bind to erythrocytes was modified by such treatment. This result is in agreement with the further finding that the SH group of cysteine is not involved in toxin interaction with its binding "receptor" (cholesterol). The blockade of the SH group very likely produced a steric hindrance to toxin fixation to the cholesterol embedded in the erythrocyte membrane. Such a hindrance does not take place when the toxin interacts with free cholesterol. The isolation of toxin-sterol complexes in liquid phase was also reported by Geoffroy and Alouf (1983), who separated and identified [$^3$H]cholesterol-ALV complexes by sucrose gradient ultracentrifugation. The complexes formed were relatively heterogeneous in size. Heat-denaturated toxin did not bind cholesterol, indicating that the native structure is essential for binding. ILO-cholesterol complexes were also separated by centrifugation at 25,000*g* (Vazquez-Boland *et al*., 1989).

## Stoichiometry

The limited solubility of cholesterol and other sterols in aqueous phase, the non-uniform nature of their dispersions in water, and their tendency to stick to solid surfaces, among other reasons, has made it difficult to obtain information on the stoichiometry and thermodynamics of toxin-sterol interaction. Cholesterol has a maximum solubility in aqueous solutions of 1.8 μg/ml (4.7 μM) and undergoes a reversible self-association at the critical micellar concentration (CMC) of approximately 27–44 nM at 25°C (see Geoffroy and Alouf, 1983). Such cholesterol aggregates are heterogeneous in size and rod-shaped, each containing about 260–360 cholesterol molecules (Smyth and Duncan, 1978). Ideally, the study of toxin-cholesterol interactions should be performed at concentrations below the CMC to ensure proper stoichiometry with cholesterol molecules dispersed as monomers rather than aggregates. Aggregate status appears to have been the case in the studies on SLO (Prigent and Alouf, 1976; Badin and Denne, 1978), CLO (Cowell and Bernheimer, 1978), and PFO (Hase *et al*., 1976), as cholesterol concentrations found to inhibit 1 HU ranged from 12 to 25 nmol, corresponding to a molar cholesterol-toxin ratio of about 500–1,000. The number of cholesterol molecules reportedly required to neutralize a single molecule of a given CDC ranged from 170 to 1 x $10^6$ (Smyth and Duncan, 1978). By using a dilution technique involving sterol solutions in absolute ethanol, Geoffroy and Alouf (1983) avoided dealing with micellar solutions and were able to determine for the first time a linear inhibition "titration" curve of ALV by cholesterol. The stoichiometry was found practically equimolar (about 1.6 molecules of cholesterol "neutralized" by 1 molecule of ALV).

## CYTOLYTIC AND MEMBRANE-DAMAGING EFFECTS

The most striking biological property of the toxins reviewed here is their potent lytic activity toward all mammalian and other eukaryotic cells so far tested. Lysis results from the disorganization or disruption of the cytoplasmic membrane of target cells. The intracellular organelles are also disrupted. The membrane-damaging effects are also reflected by alterations in the permeability and integrity of cholesterol-containing artificial lipid model membranes (liposomes, black films, monolayers) (Alouf *et al*., 1984; Korchev *et al*., 1998; Menestrina *et al*., 1990).

### Cell lysis markers

The lytic process triggered by the toxins reviewed here and other cytolysins is classically investigated and monitored by the measurement of the *in vitro* release of intracellular components (from cell cytoplasm or from the disrupted organelles) into the incubation medium by the toxin-damaged cells (Alouf, 1977; Smyth and Duncan, 1978; Alouf and Palmer, 1999). The release of radiolabeled, colored, or fluorescent markers entrapped in resealed erythrocyte vesicles (Buckingham and Duncan, 1983) or in liposomes (Duncan, 1984; Menestrina *et al*., 1990) has been also used in the studies of toxin-induced damage of these vesicles.

The spectrophotometric determination of hemoglobin (Hb) is the most usual method employed in the monitoring of erythrocyte lysis (hemolysis) and its kinetics (Alouf and Geoffroy, 1988). The release of $K^+$ or $^{86}Rb$ from toxin-treated erythrocytes was also used as a marker of cell lysis by TLY (Blumenthal and Habig, 1984) or by SLO (Smyth and Duncan, 1978). A highly sensitive approach is the measurement by a bioluminescence method of the release of ATP from erythrocytes and other cells devised by Fehrenbach *et al*. (1980) and used for the study of the kinetics of erythrocyte lysis by SLO (Niedermeyer, 1985). A comparative study of Hb and ATP release showed that the latter was a much more sensitive indicator of SLO-induced lysis ($10^{-11}$–$10^{-12}$ M ATP) than Hb.

Platelet lysis by SLO and ALV was followed by the assay of released serotonin, lactate dehydrogenase, and other enzymes (Launay and Alouf, 1979; Launay *et al*., 1984). Membrane damage of human lung fibroblasts by SLO, PFO, and ALV was assessed by the leakage of three different-sized cytoplasmic markers (Thelestam and Möllby, 1980; Thelestam *et al*., 1981). The cells were first preloaded with [$^{14}$C]amino isobutyric acid ($M_r$ 103) or treated with radiolabeled uridine to obtain either a labeled nucleotide ($M_r$ < 1,000) or RNA ($M_r$ < 200,000) before toxin challenge. The release of these markers was monitored for the study of the time course of the lytic process and for the estimation of the size of the functional "pores" (whatever their physical reality) elicited in the damaged membrane (Thelestam and Möllby, 1983). In this respect, references to "pores," "channels," or "lesions" are meant to indicate functional entities with the potential to allow passage of molecules up to a certain size (Buckingham and Duncan, 1983).

Preloaded [$^3$H]choline or [$^{35}$S]methionine Lettre murine tumor cells were used as precursors for labeled intracellular markers for the study of the mechanism of lysis of these cells by PFO and other toxins (Menestrina *et al*., 1990).

### Membrane cholesterol as the toxin target

Several lines of experimental evidence suggest that cholesterol molecules embedded in the lipid bilayer of the cytoplasmic membrane of eukaryotic cells constitute the binding site of the toxins:

i. The toxins are inactivated by cholesterol and can no longer bind target cells (Alouf, 1980; Ohno-Iwashita *et al*., 1986).
ii. The toxins have no lytic effects on prokaryotic cells (bacterial protoplasts and spheroplasts), which lack cholesterol in their cytoplasmic membranes. However, parasitic mycoplasma cells that contain cholesterol are bound and damaged by the toxins. Saprophytic mycoplasma cells, on the other hand, in which carotenol replaces cholesterol in their membranes, are not damaged by these toxins (Smyth and Duncan, 1978; Rottem *et al*., 1976, 1990). Incubation of these cells with a cholesterol-Tween mixture reestablished their ability to bind CLO. Furthermore, the treatment of human erythrocyte membranes and *Acholeplasma laidlawii* cells containing cholesterol with cholesterol oxidase abolished their ability to bind CLO and to inhibit hemolysis (Cowell and Bernheimer, 1978).
iii. The lytic activity of the toxins is abrogated by incubation with erythrocyte membranes; in membrane extracts, only those lipid fractions that contain cholesterol inactivate the toxins (Hase *et al*., 1976; Smyth and Duncan, 1978). Similarly, *A. laidlawii* cells grown in the presence of cholesterol inhibited the hemolytic activity of CLO, but not *A. laidlawii* cells grown in the absence of cholesterol.
iv. The pretreatment of erythrocyte membranes with agents that are known to bind cholesterol such as polyene antibiotics and saponins prevented the

inhibition of the hemolytic activity of CLO and SLO (Shany *et al*., 1974). In addition, the treatment of nucleated mammalian cells (L cells and HeLa cells) with inhibitors of cholesterol synthesis, such as 20α-hydroxysterol or 25-hydroxysterol, significantly reduced SLO binding and abolished cell susceptibi-lity to the lytic effect of the toxin; the incubation of refractory cells with serum or cholesterol restored their sensitivity to SLO (Duncan and Buckingham, 1980).

v. The partial evulsion of cholesterol (about 30%) from human erythrocytes decreased PFO binding and cell susceptibility to lysis (Mitsui *et al*., 1982), whereas experimentally cholesterol-enriched erythrocytes exhibited increased sensitivity to lysis by SLO (Linder and Bernheimer, 1984).

vi. Only cholesterol-containing phospholipid (but not pure phospholipid) vesicles or films are bound and disrupted by the toxins (Alouf *et al*., 1984).

Despite the plethora of evidence that CDCs interact directly with cholesterol in the host membrane, the exact nature of this interaction is still in debate. While most CDCs bind to all cholesterol-containing membranes, albeit with different affinities, ILY appears to be particularly specific for human-derived cells (Nagamune *et al*., 1996), suggesting that cholesterol is unlikely to be involved in membrane recognition by this toxin. Recent findings by Giggings *et al*. (2003) indicated that depletion of cholesterol from erythrocyte membranes stalled each of PFO, SLO, and ILY at the prepore complex. In addition, these authors showed that depletion of membrane cholesterol, while decreasing PFO binding approximately 10-fold, did not affect the binding of SLO or ILY to host membranes. Therefore, in contrast to implications that cholesterol is the major host cell receptor for CDCs, these results suggest that cholesterol plays its primary role in facilitating the structural changes in CDCs that lead to membrane insertion.

## ELECTRON MICROSCOPY STUDIES OF TOXIN INTERACTION WITH BIOLOGICAL AND ARTIFICIAL TARGET MEMBRANES

As described in detail in the next four chapters, toxin molecules bind as monomers to the membrane surface with subsequent oligomerization (about 50 monomers). This process is reflected by arc- and ring-shaped structures surrounding large pores generated by this process. As early as 1966 such ring- and arc-shaped structures were observed by electron microscopy on the suface of erythrocytes treated with SLO (Dourmashkin and Rosse, 1966). This observation led to extensive studies over the following 35 years on these and other eukaryotic cells challenged with SLO (Duncan and Schlegel, 1975; Bhakdi *et al*., 1985; Niedermeyer, 1985; Sekiya *et al*., 1993, 1996; Palmer *et al*., 1998), PFO (Smythe *et al*., 1975; Mitsui *et al*., 1979), CLO (Cowell *et al*., 1978), TLY (Alving *et al*., 1979), LLO (Jacobs *et al*., 1998), BLY (Sekiya *et al*., 1998), and PLY (Morgan *et al*., 1994; Korchev *et al*., 1998). Interestingly, the formation of similar arc- and ring-shaped structures at the surface of target cells was observed in the case of the eukaryotic sea anemone *Metridium senile*, which produces a cholesterol-binding cytolysin sharing several biological properties of bacterial CDCs (Bernheimer *et al*., 1979). Furthermore, artificial cholesterol-containing lipid vesicles (liposomes) treated with some of these CDCs also showed similar arc- and ring-shaped structures (Morgan *et al*., 1995; Korchev *et al*., 1998).

## CONCLUSION

Important progress has been made in the past five years with regard to the mechanisms of toxin insertion and oligomerization in target cells in relation to membrane cholesterol (Shatursky *et al*., 1999; Gilbert *et al*., 1999, 2000; Palmer, 2001; Tweten *et al*., 2001; Giddings *et al*., 2003) and the genetics and structural characteristics of these toxins (see following chapters). However, the role of certain CDCs in disease, particularly in inflammatory processes and probably in sepsis, remains poorly understood. Moreover, the involvement of CDCs in host-signaling pathways still requires further investigation. Finally, the potential use of certain CDCs as potent permeabilization probes in cell biology or as vaccines or novel therapeutical agents remains an important and promising issue.

## REFERENCES

Abdel Ghani, E.M., Weis, S., Walev, I., Kehoe, M., Bhakdi, S. and Palmer, M. (1999). Streptolysin O: inhibition of the conformational change during membrane binding of the monomer prevents oligomerization and pore formation. *Biochem*. **38,** 15204–15211.

Allen, A.G., Bolitho, S., Lindsay, H., Khan, S., Bryant, C., Norton, P., Ward, P., Leigh, J., Morgan, J., Riches, H., Eastty, S. and Maskell, D. (2001). Generation and characterization of a defined mutant of *Streptococcus suis* lacking suilysin. *Infect. Immun*. **69,** 2732–2735.

Alouf J. E. (1977). Cell membranes and cytolytic bacterial toxins. In: *Specificity and Action of Animal, Bacterial, and Plant Toxins* (ed. P. Cuatrecasas), pp. 211–270. Chapman and Hall, London.

Alouf, J.E. (1980). Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther*. **11,** 211–270.

Alouf J. E. (1999). Introduction to the family of the structurally-related cholesterol-binding cytolysins (sulfhydryl-activated toxins). In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J. E. Alouf and J. H. Freer), pp. 443–456, Academic Press, London.

Alouf, J.E. (2000). Cholesterol-binding cytolytic protein toxins. *Int. J. Med. Microbiol*. **290,** 351–356.

Alouf J. E. (2001). Pore-forming bacterial protein toxins. In: *Pore Forming Toxins* (ed. F. G. van der Goot), pp. 1–14. Springer, Berlin.

Alouf, J.E. and Geoffroy, C. (1979). Comparative effects of cholesterol and thiocholesterol on streptolysin O. *FEMS Microbiol. Lett*. **6,** 413–416.

Alouf J.E. and Geoffroy C. (1988). Production, purification, and assay of streptolysin O. *Methods Enzymol*. **165,** 52–59.

Alouf, J.E., Kiredjian, M. and Geoffroy, C. (1977). Purification de l'hémolysine thiol-dépendante extracellulaire de *Bacillus alvei. Biochim*. **59,** 329–336.

Alouf J.E. and Palmer M. (1999). Streptolysin O. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J. E. Alouf and J. H. Freer), pp. 459–473. Academic Press, London.

Alouf, J.E., Geoffroy, C., Pattus, F. and Verger, R. (1984). Surface properties of bacterial sulfhydryl-activated cytolytic toxins. Interaction with monomolecular films of phosphatidylcholine and various sterols. *Eur. J. Biochem*. **141,** 205–210.

Alving, C.R., Habig, W.B., Urban, K.A. and Hardegree, M.C. (1979). Cholesterol-dependent tetanolysin damage to liposomes. *Biochim. Biophys. Acta* **551,** 224–228.

Ashdown, L.R. and Koehler, J.M. (1990). Production of hemolysin and other exoenzymes by clinical isolates of *Pseudomonas pseudomallei. J. Clin. Microbiol*. **28,** 2331–2334.

Badin, J. and Denne, M.-A. (1978). A cholesterol fraction for streptolysin O binding on cell membranes and lipoproteins. *Cell. Molec. Biol*. **22,** 133–143.

Bernheimer A. W. (1976). Sulfhydryl activated toxins. In: *Mechanisms in Bacterial Toxinology* (ed. A.W. Bernheimrer), pp. 85–97. John Wiley, New York.

Bernheimer, A.W. and Avigad, L.S. (1970). Streptolysin O activation by thiols. *Infect. Immun*. **1,** 509–510.

Bernheimer, A.W., Avigad, L.S. and Kim, K-S. (1979). Comparison of metridiolysin from the sea anemone with thiol-activated cytolysins from bacteria. *Toxicon* **17,** 69–75.

Bernheimer, A.W. and Grushoff, P. (1967a). Cereolysin: production, purification, and partial characterization. *J. Gen. Microbiol*. **46,** 143–150.

Bernheimer, A.W. and Grushoff, P. (1967b). Extracellular hemolysins of aerobic sporogenic bacilli. *J. Bacteriol*. **93,** 1541–1543.

Berthou, L., Corvaia, N., Geoffroy, C., Mutel, V., Launay, J-M. and Alouf, J.E. (1992). The phosphoinositide pathway of lymphoid cells: labeling after permeabilization by alveolysin, a bacterial sulfhydryl-activated cytolysin. *Eur. J. Cell. Biol*. **58,** 377–82.

Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985). Mechanism of membrane damage to cell membranes by streptolysin O. *Infect. Immun*. **47,** 52–60.

Bhakdi, S., Bayley, H., Valeva, A., Walev, I., Walker, B., Kehoe, M. and Palmer, M. (1996). Staphylococcal alpha-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins. *Arch. Microbiol*. **165,** 73–79.

Billington, S.J., Jost, B.H., Cuevas, W.A., Bright, K.R. and Songer, J.G. (1997). The *Arcanobacterium* (*Actinomyces*) hemolysin, pyolysin, is a novel member of the thiol-activated cytolysin family. *J. Bacteriol*. **179,** 6100–6106.

Billington, S.J., Jost, B.H. and Songer J.G. (2000). Thiol-activated cytolysins: structure, function, and role in pathogenesis. *FEMS Microbiol. Lett*. **182,** 197–205.

Billington, S.J., Songer, J.G. and Jost, B.H. (2002). The variant undecapeptide sequence of the *Arcanobacterium pyogenes* hemolysin, pyolysin, is required for full cytolytic activity. *Microbiology* **148,** 3947–3954.

Blumenthal, R. and Habig, W.H. (1984). Mechanisms of tetanolysin-induced membrane damage: studies with black lipid membranes. *J. Bacteriol*. **157,** 321–323.

Boulnois, G.J., Paton, J.C., Mitchell, T.J. and Andrew, P.W. (1991). Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae. Molec. Microbiol*. **5,** 2611–2616.

Buckingham, L. and Duncan, J.L. (1983). Approximate dimensions of membrane lesions produced by streptolysin S and streptolysin O. *Biochim. Biophys. Acta* **729,** 115–122.

Burn, C.G. (1934). Unidentified Gram-positive bacillus associated with meningo-encephalitis. *Proc. Soc. Exp. Biol. Med*. **31,** 1095.

Charland, N., Nizet, V., Rubens, C.E., Kim, K.S., Lacouture, S. and Gottschalk, M. (2000). *Streptococcus suis* serotype 2 interactions with human brain microvascular endothelial cells. *Infect. Immun*. **68,** 637–643.

Cohen, B., Perkins, M.E. and Putterman, S. (1940). The reaction between hemolysin and cholesterol. *J. Bacteriol*. **39,** 59–60.

Cole, R. (1914). Pneumococcus hemotoxin. *J. Exp. Med*. **20,** 346–362.

Coudron, P.E., Payne, J.M. and Markowitz, S.M. (1991). Pneumonia and empyema infection associated with a *Bacillus* species that resembles *B. alvei. J. Clin. Microbiol*. **29,** 1777–1779.

Cowell, J.L. and Bernheimer, A.W. (1978). Role of cholesterol in the action of cereolysin membrane. *Arch. Biochem. Biophys*. **190,** 306–310.

Cowell, J.L., Kim, K.S and Bernheimer, A.W. (1978). Alteration by cereolysin of the structure of cholesterol-containing membranes. *Biochim. Biophys. Acta* **507,** 230–241.

Darji, A., Niebuhr, K., Hense, M., Wehland, J., Chakraborty, T. and Weiss, S. (1996). Neutralizing monoclonal antibodies against listeriolysin: mapping of epitopes involved in pore formation. *Infect. Immun*. **64,** 2356–2358.

de los Toyos, J.R., Mendez, F.J., Aparicio, J.F., Vázquez, F., del Mar García Suárez, M., Fleites, A., Hardisson, C., Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1996). Functional analysis of pneumolysin by use of monoclonal antibodies. *Infect. Immun*. **64,** 480–484.

Ding, H. and Lämmler, C. (1996). Purification and further characterization of a hemolysin of *Actinomyces pyogenes. J. Vet. Med. B*. **43,** 179–188.

Domann, E. and Chakraborty, T. (1989). Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype ½a strain. *Nucleic Acids Res*. **17,** 6406.

Dourmashkin, R.R. and Rosse, W.F. (1966). Morphologic changes in the membranes of red blood cells undergoing hemolysis. *Am. J. Med*. **41,** 699–710.

Dramsi, S. and Cossart, P. (2002). Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. *J. Cell. Biol*. **156,** 943–946.

Duncan, J.L. (1984). Liposomes as membrane models in studies of bacterial toxins. *J. Toxicol. Toxin. Rev*. **3,** 1–51.

Duncan, J.L. and Buckingham L. (1980). Resistance to streptolysin O in mammalian cells treated with oxygenated derivatives of cholesterol. Cholesterol content of resistant cells and recovery of streptolysin O sensitivity. *Biochim. Biophys. Acta*. **603,** 278–287.

Duncan, J.L. and Schlegel, R. (1975). Effect of streptolysin O on erythrocyte membranes, liposomes, and lipid dispersions. A protein-cholesterol interaction. *J. Cell Biol*. **67,** 160–173.

Ehrlich, P. (1898). Discussion during Gesellschaft der Charite Arzte. *Berlin. Klin.Wsch*. **35,** 273.

Eisenberg, P. (1907). Sur les hémolysines des anaérobies. *C. R. Soc. Biol*. **62,** 537–539.

Fehrenbach, F.J., Huser, H. and Jaschinski, C. (1980). Measurement of bacterial cytolysins with a highly sensitive kinetic method. *FEMS Microbiol. Lett*. **7,** 285–286.

Figueiredo, P.M., Catani, C.F. and Yano, T. (2003). Thiol-independent activity of a cholesterol–binding enterohemolysin produced by enteropathogenic *Escherichia coli*. *Braz. J. Med. Biol. Res*. **36,** 1495–1499.

Funk, P.G., Staats, J.J., Howe, M, Nagaraja, T.G. and Chengappa, M.M. (1996). Identification and partial purification of an *Actinomyces pyogenes* hemolysin. *Vet. Microbiol*. **50,** 129–142.

Geoffroy, C. and Alouf, J.E. (1982). Interaction of alveolysin, a sulfhydryl-activated bacterial cytolytic toxin, with thiol group reagents and cholesterol. *Toxicon* **20,** 239–241.

Geoffroy, C. and Alouf, J.E. (1983). Selective purification by thiol-exchange chromatography of alveolysin, a sulfhydryl-activated toxin of *Bacillus alvei*. *J. Biol. Chem*. **258,** 9968–9972.

Geoffroy, C., Gaillard, J.-L., Alouf, J.E. and Berche, P. (1987). Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect. Immun*. **55,** 1641–1646.

Geoffroy, C., Gaillard, J.-L., Alouf, J.E. and Berche, P. (1989). Production of thiol-dependent hemolysins by *Listeria monocytogenes* and related species. *J. Gen. Microbiol*. **35,** 481–487.

Geoffroy, C., Mengaud, J., Alouf, J.E. and Cossart, P. (1990). Alveolysin, the thiol-dependent toxin of *Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin and streptolysin O, and contains a single cysteine. *J. Bacteriol*. **172,** 7301–7305.

Giddings, K.S., Johnson, A.E. and Tweten, R.K. (2003). Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *Proc. Natl. Acad. Sci. USA* **100,** 11315–11320.

Gilbert, R.J., Jiménez, J.L., Chen, S., Tickle, I.J., Rossjohn, J., Parker, M., Andrew, P.W. and Saibil, H.R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*. *Cell* **97,** 647–655.

Gilbert, R.J., Jiménez, J.L., Chen, S., Andrew, P.W. and Saibil, H.R. (2000). Structural basis of pore formation by cholesterol-binding toxins. *Int. J. Med. Microbiol*. **290,** 389–394.

Guillaumie, M. (1950). Hémolysines bactériennes et anti hémolysines. *Ann. Inst. Pasteur* **79,** 661–670.

Guillaumie, M. and Kréguer, A. (1950). Nouvelles recherches sur les hémolysines oxydables. *Ann. Inst. Pasteur* **78,** 467–480.

Haas, A., Dumbsky, M. and Kreft, J. (1992). Listeriolysin genes: complete nucleotide sequence of *ilo* from *Listeria ivanovii* and of *lso* from *Listeria seeligeri*. *Biochim. Biophys. Acta* **1130,** 81–84.

Halbert, S.P., Cohen, B. and Perkins, M.E. (1946). Toxic and immunological properties of pneumococcal hemolysin. *Bull. Johns Hopkins Hosp*. **78,** 340–359.

Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S. and Matsuda, M. (1992). Production, purification, and characterization of botunolysin, a thiol-activated hemolysin of *Clostridium botulinum*. *Infect. Immun*. **60,** 71–78.

Hardegree, M.E. (1965). Separation of neurotoxin and hemolysin of *Clostridium tetani*. *Proc. Soc. Exp. Biol. Med*. **119,** 405–488.

Harvey, P.C. and Faber, J.E. (1941). Some biochemical reactions of the *Listerella* group. *J. Bacteriol*. **41,** 45–46.

Hase, J., Mitsui, K. and Shonaka, E. (1976). *Clostridium perfringen* exotoxins. IV. Inhibition of toxin-induced hemolysis by steroids and related compounds. *Jap. J. Exp. Med*. **46,** 45–50.

Hatheway, C.L. (1990). Toxigenic clostridia. *Clin. Microbiol. Rev*. **3,** 66–98.

Herbert, D. and Todd, A.W. (1941). Purification and properties of a hemolysin produced by group A hemolytic streptococci (streptolysin O). *Biochem. J*. **35,** 1124–1139.

Herter, C.A. (1906–1907). On bacterial processes in the intestinal tract in some cases of advanced anemia with special reference to infection with B.aerogenes. *J. Biol. Chem*. **2,** 1–70.

Heuck, A.P., Hotze, E.M., Tweten, R.K. and Johnson, A.E. (2000). Mechanism of membrane insertion of a multimeric barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. *Molec. Cell* **6,** 1233–1242.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2001). Beta-barrel pore-forming toxins: intriguing dimorphic proteins. *Biochemistry* **40,** 9065–9073.

Howard, J.G. (1953). The hemolysin of *Clostridium hemolyticum*. *Brit. J. Exp. Path*. **34,** 564–567.

Jacobs, A.A.C, Loffen, P.L.W., van den Berg, A.J.G. and Storm, P.K. (1994). Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. *Infect. Immun*. **62,** 1742–1748.

Jacobs, A.A., van den Berg A.J. and Loeffen, P.L. (1996). Protection of experimentally infected pigs by suilysin, the thiol-activated hemolysin of *Streptococcus suis*. *Vet. Rec*. **139,** 225–228.

Jarvill-Taylor, K. and Minion, F.K. (1995). The effect of thiol-active compounds and sterols on the membrane-associated hemolysin of *Mycoplasma pulmonis*. *FEMS Microbiol. Lett*. **128,** 213–218.

Jenkins, E.M., Njoku-Obi, A.N. and Adams, E.W. (1964). Purification of the soluble hemolysin of *Listeria monocytogenes*. *J. Bacteriol*. **88,** 418–424.

Johnson, M.K. (1977). Cellular location of pneumolysin. *FEMS Microbiol. Lett*. **2,** 243–245.

Johnson, M.K., Geoffroy, C. and Alouf, J.E. (1980). Binding of cholesterol by sulfhydryl-activated cytolysins. *Infect. Immun*. **27,** 97–101.

Jost, B.H., Songer, J.G. and Billington, S.J. (1999). An *Arcanobacterium* (*Actinomyces*) *pyogenes* mutant deficient in production of the pore-forming cytolysin pyolysin has reduced virulence. *Infect. Immun*. **67,** 1723–1728.

Jost, B.H., Trinh, H.T., Songer, J.G. and Billington, S.J. (2003). Immunization with genetic toxoids of the *Arcanobacterium pyogenes* cholesterol-dependent cytolysin, pyolysin, protects mice against infection. *Infect. Immun*. **71,** 2966–2969.

Kehoe, M.A., Miller, L., Walker, J.A. and Boulnois, G.J. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. *Infect. Immun*. **55,** 3228–3232.

Korchev, Y.E., Bashford, C.D., Pederzolli, C., Pasternak, C.A., Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1998). A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers. *Biochem. J*. **329,** 571–577.

Kim, B.S. and Kim, J.S. (2002). Cholesterol induces oligomerization of *Vibrio vulnificus* cytolysin specifically. *Exp. Mol. Med*. **34,** 239–242.

Kingdon, G.C. and Sword, C.P. (1970). Effects of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. *Infect. Immun*. **1,** 356–362.

Lalonde, M., Segura, M., Lacouture, S. and Gottschalk, M. (2000). Interactions between *Streptococcus suis* serotype 2 and different epithelial cell lines. *Microbiology* **146,** 1913–1921.

Launay, J.M. and Alouf, J.E. (1979). Biochemical and ultrastructural study of the disruption of blood platelets by streptolysin O. *Biochim. Biophys. Acta* **556,** 278–291.

Launay, J.M., Geoffroy, C., Costa, J.L. and Alouf, J.E. (1984). Purified SH-activated toxins (streptolysin O, alveolysin): new tools for

determination of platelet enzyme activities. *Thromb. Res*. **33,** 189–196.

Leimester-Wächter, M. and Chakraborty, T. (1989). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes, Listeria ivanovii*, and *Listeria seeligeri. Infect. Immun*. **57,** 2350–2357.

Lesieur, C., Vecsey-Semjn, B., Abrami, L., Fivaz, M., van der Goot, F.G. (1997). Membrane insertion: the strategy of toxins. *Mol. Membr. Biol*. **14,** 45–64.

Libman, E. (1906). A pneumococcus producing a peculiar form of hemolysis. *Proc. NY. Pathol. Soc*. **5,** 168.

Linder, R. and Bernheimer, A.W. (1984). Action of bacterial cytotoxins on normal mammalian cells and cells with altered membrane lipid composition. *Toxicon* **22,** 641–651.

Lovell, R. (1937). Studies on *Corynebacterium pyogenes*, with special reference to toxin production. *J. Pathol. Bacteriol*. **45,** 339–355.

Lovell, R. (1939). The *Corynebacterium pyogenes* antitoxin content of animal sera. *J. Pathol. Bacteriol*. **49,** 329–338.

Lovell, R. (1944). Further studies on the toxin of *Corynebacterium pyogenes. J. Pathol. Bacteriol*. **56,** 525–529.

Lucain, C. and Piffaretti, J.-C. (1977). Characterization of the hemolysins of different serotypes of *Clostridium tetani. FEMS Microbiol. Lett*. **1,** 231–234.

Lun, S., Perez-Casal, J., Connor, W. and Willson, P.J. (2003). Role of suilysin in the pathogenesis of *Streptococcus suis* capsular serotype 2. *Microbial Path*. **34,** 27–37.

Menestrina, G., Bashford, C.L. and Pasternak, C.A. (1990). Pore-forming toxins: experiments with *S. aureus* α-toxin, *C. perfringens* ϕ-toxin, and *E. coli* hemolysin in lipid bilayers, liposomes, and intact cells. *Toxicon* **28,** 477–491.

Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J.-L. and Cossart, P. (1987). Identification of the structural gene encoding the SH-activated hemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. *Infect. Immun*. **55,** 3225–3227.

Marmorek, A. (1902). La toxine streptococcique. *Ann. Inst. Pasteur* **16,** 169–178.

Michel, E., Reich, K.A., Favier, R., Berche, P. and Cossart, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitution in listeriolysin O. *Molec. Microbiol*. **4,** 2167–2178.

Mita, T. (1934). Untersuchungen über den Bacillus Histolyticus. *Jap. J. Exp. Med*. **12,** 285–297.

Mitsui, N., Mitsui, K. and Hase, J. (1980). Purification and some properties of tetanolysin. *Microbiol. Immunol*. **7,** 221–229.

Mitsui, K., Sekiya, T, Nozawa, Y. and Hase, J. (1979). Alteration of human erythrocyte plasma membrane by perfringolysin O as revealed by freeze-fracture electron microscopy. *Biochim. Biophys. Acta* **554,** 68–75.

Mitsui, K., Saeki, Y. and Hase, J. (1982). Effects of cholesterol evulsion on susceptibility of perfringolysin O of human erythrocytes. *Biochim. Biophys. Acta* **686,** 307–313.

Morgan, P.J., Hayman, S.C., Byron, O., Andrew, W., Mitchell, T.J. and. Rowe, A.J. (1994). Modeling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. *J. Biol. Chem*. **269,** 25315–25320.

Morgan, P.J., Hyman, S.C., Rowe, A.J., Mitchell, T.J., Andrew, P.W. and Saibil, H.R. (1995). Subunit organization and symmetry of pore-forming, oligomeric pneumolysin. *FEBS Lett*. **371,** 77–80.

Morgan, P.J., Andrew, P.W., Mitchell, T.J. (1996). Thiol-activated cytolysins. *Rev. Med. Microbiol*. **7,** 221–229.

Moussa, R.S. (1958). Complexity of toxins from *Clostridium septicum* and *Clostridium chauvoei. J. Bacteriol*. **76,** 538–545.

Nagamune, H. (1997). Streptococcal cytolysins. *Seikagaku* **69,** 343–348.

Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R.A, Tsuji, A. and Matsuda, Y. (1996). Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abcess. *Infect. Immun*. **64,** 3093–3100.

Nagamune, H., Whiley, R.A., Goto, T., Inai, Y., Maeda, T., Hardie, J.M. and Kourai, H. (2000). Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with *Streptococcus intermedius. J. Clin. Microbiol*. **38,** 220–226.

Nakamura, M., Sekino, N., Iwamoto, M. and Ohno-Iwashita, Y. (1995). Interaction of toxin (perfringolysin O), a cholesterol-binding cytolysin, with liposomal membranes: change in the aromatic side chains upon binding and insertion. *Biochem*. **34,** 6513–6520.

Neill, J.B. (1926a). Studies on the oxidation and reduction of immunological substances. I. Pneumococcus hemotoxin. *J. Exp. Med*. **44,** 199–213.

Neill, J.B. (1926b). Studies on the oxidation and reduction of immunological substances. II. The hemotoxin of the Welch bacillus. *J. Exp. Med*. **44,** 215–226.

Neill, J.B. (1926c). Studies on the oxidation and reduction of immunological substances. III. Tetanolysin. *J. Exp. Med*. **44,** 227–240.

Neill, J.B and Mallory, T.B. (1926). Studies on the oxidation and reduction of immunological substances. IV. Streptolysin. *J. Exp. Med*. **44,** 241–260.

Neill, J.B and Fleming, W.L. (I927). Studies on the oxidation and reduction of immunological substances. VI. The "reactivation" of the bacteriolytic activity of oxidized pneumococcus extracts. *J. Exp. Med*. **46,** 263–277.

Niedermeyer, W. (1985). Interaction of streptolysin O with membranes: kinetic and morphological studies on erythrocytes membranes. *Toxicon* **23,** 425–435.

Njoku-Obi, A.N., Jenkins, E.M., Njoku-Obi, J.C., Adams, J. and Covington, V. (1963). Production and nature of *Listeria monocytogenes* hemolysins. *J.Bacteriol*. **86,** 1–8.

Norton, P.M., Rolph, C., Ward, P.N., Bentley, R.W. and Leigh, J.A. (1999). Epithelial invasion and cell lysis by virulent strains of *Streptococcus suis* is enhanced by the presence of suilysin. *FEMS Immunol. Med. Microbiol*. **26,** 25–35.

Oakley, C.L., Warrack, G.H. and Clarke, P.H. (1947). The toxins of *Clostridium oedematiens* (*Cl. novyi*). *J.Gen. Microbiol*. **1,** 91–107.

Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K., Kawasaki, H. and Ando, S. (1986). Cold-labile hemolysin produced by limited proteolysis of ϕ-toxin from *Clostridium perfringens. Biochemistry* **25,** 6048–6053.

Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K, Ando, S. and Nagai, K. (1988). Protease-nicked theta toxin of *Clostridium perfringens*, a new membrane probe with no cyotolytic effect, reveals two classes of cholesterol as toxin-binding sites on sheep erythrocytes. *Eur J. Biochem*. **176,** 95–101.

Okumura, K., Hara, A., Tanaka, T., Nishigushi, T., Minamide, W., Igarashi, H. and Yotsudo, T. (1994). Cloning and sequencing the streptolyson O genes of group C and G streptococci. *DNA Seq*. **4,** 325–28.

Palmer, M. (2001). The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon* **39,** 1681–1689.

Palmer, M., Vulicevic, I., Saweljew, P., Valeva, A., Kehoe, M. and Bhakdi, S. (1998). Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochem*. **37,** 2378–2383.

Pendelton, I.R., Bernheimer, A.W. and Grushoff, P. (1973). Purification and partial characterization of hemolysins from *Bacillus thuringiensis. J. Invertebrate Pathol*. **21,** 131–135.

Pinkney, M., Beachey, E. and Kehoe, M. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun*. **57,** 2553–2558.

Prigent, D. and Alouf, J.E. (1976). Interaction of streptolysin O with sterols. *Biochim. Biophys. Acta* **443,** 288–300.

Rakotobe, F. and Alouf J. E. (1984). Purification and properties of thuringolysin, a sulfhydryl-activated cytolytic toxin of *Bacillus thuringensis*. In: *Bacterial Protein Toxins*. (eds. J.E. Alouf, F. J. Fehrenbach, J. H. Freer and J. Jeljaszewicz), pp. 265, Academic Press, New York.

Ramachandran, R., Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2002). Structual insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. *Nat. Struct. Biol*. **9,** 823–827.

Ramachandran, R., Tweten, R.K. and Johnson, A.E. (2004). Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit strand alignment. *Nat. Struct. Molec. Biol*. **11,** 697–705.

Reboli, A.C., Bryan, S.C. and Farrar, W.E. (1989). Bacteremia and hip prosthesis caused by *Bacillus alvei. J. Clin. Microbiol*. **27,** 1395–1396.

Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89,** 685–692.

Rottem, S., Hardegree, M.C., Grabowski, M.R., Fornwald, R. and Barile, M.F. (1976). Interaction between tetanolysin and mycoplasma cell membrane. *Biochim. Biophys. Acta* **455,** 879–888.

Rottem, S., Groover, K., Habig, W.H., Barile, M.F. and Hardegree, M.C. (1990). Transmembrane diffusion channels in *Mycoplasma gallisepticum* induced by tetanolysin. *Infect. Immun*. **58,** 598–602.

Rudnick, S.T., Jost, B.H., Songer, J.G. and Billington, S.J. (2003). The gene encoding pyolysin, the pore-forming toxin of *Arcanobacterium pyogenes*, resides within a genomic islet flanked by essential genes. *FEMS Microbiol. Lett*. **225,** 241–247.

Rutter, J.M. and Collie, J.F. (1969). Studies on the soluble antigens of *Clostridium oedematiens (Cl . novyi). J.Med.Microbiol*. **2,** 395–421.

Sato, H. (1986). Monoclonal antibodies against *Clostridium perfringens* theta-toxin (perfringolysin O). In: *Monoclonal Antibodies Against Bacteria* (eds. A.J.L. Macario and E. Conway de Macario), pp. 203–228. Academic Press, New York.

Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W. and Boulnois, G.J. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect. Immun*. **57,** 2547–2552.

Segers, R.P.A.M., Kenter, T., de Haan, L.A. and Jacobs, A.A.C. (1998). Characterization of the gene encoding suilysin from *Streptococcus suis* and expression in field strains. *FEMS Microbiol.Lett*. **167,** 255–261.

Segura, M. and Gottschalk, M. (2002). *Streptococcus suis* interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. *Infect. Immun*. **70,** 4312–4322.

Sekino-Suzuki, N., Nakamura, M., Mitsui, K. I. and Ohno-Iwashita, Y. (1996). Contribution of individual tryptophan residues to the structure and activity of θ-toxin (perfringolysin O), a cholesterol-binding cytolysin. *Eur. J. Biochem*. **241,** 941–947.

Sekiya, K., Satoh, R., Danbara, H. and Futaesaku, Y. (1993). A ring-shaped structure with a crown formed by streptolysin O on the erthrocyte membrane. *J. Bacteriol*. **175,** 5953–5961.

Sekiya, K., Satoh, R., Danbara, H. and Futaesaku, Y. (1996). Electron microscopic evaluation of a two-step theory of pore formation by streptolysin O. *J. Bacteriol*. **178,** 6998–7002.

Sekiya, K., Danbara, H., Futaesaku, Y., Haque, A., Sugimoto, N. and Matsuda, M. (1998). Formation of ring-shaped structures on erythrocyte membranes after treatment with botulinolysin, a thiol-activated hemolysin from *Clostridium botulinum. Infect. Immun*. **66,** 2987–2990.

Shannon, J.G., Ross, C.L., Koehler, T.M. and Rest, R.F. (2003) Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin. *Infect. Immun*. **71,** 3183–3189.

Shany, S., Bernheimer, A.W., Grushoff, P.S. and Kim, K.-S. (1974). Evidence for membrane cholesterol as the common binding site for cereolysin, streptolysin O, and saponin. *Cell. Mol. Biochem*. **3,** 179–186.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Shepard, L.A., Heuck, A.P., Hamman, D.B., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: an α-helical to β-sheet transition identified by fluorescence spectroscopy. *Biochem*. **37,** 14563–14574.

Shepard, L.A., Shatursky, O., Johnson, A.E. and Tweten, R.K. (2000). The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane hairpins. *Biochem*. **39,** 10284–10293.

Smyth, C. J. and Duncan, J. L. (1978). Thiol-activated (oxygen-labile) cytolysins. In: *Bacterial Toxins and Cell Membranes* (eds. J. Jeljaszewicz and T. Wadström), pp. 129–183. Academic Press, London.

Smythe, C.V. and Harris, T.N. (1940). Some properties of a hemolysin produced by group A hemolytic streptococci. *J. Immunol*. **38,** 283–300.

Sugimoto, N., Haque, A., Horiguchi, Y. and Matsuda, M. (1995). Coronary vasoconstriction is the most probable cause of death of rats intoxicated with botulinolysin, a hemolysin produced by *Clostridium botulinum. Toxicon* **33,** 1215–1230.

Sugimoto, N., Haque, A., Horiguchi, Y. and Matsuda, M. (1997). Botulinolysin, a thiol-activated hemolysin produced by *Clostridium botulinum*, inhibits endothelium-dependent relaxation of rat aortic ring. *Toxicon* **35,** 1011–1023.

Takamatsu, D., Osaki, M. and Sekizaki, T. (2002). Evidence for lateral transfer of the suilysin gene region of *Streptococcus suis. J. Bacteriol*. **184,** 2050–2057.

Thelestam, M. and Möllby, R. (1980). Interaction of streptolysin O from *Streptococcus pyogenes* and theta toxin from *Clostridium perfringens* with human fibroblasts. *Infect. Immun*. **29,** 863–877.

Thelestam, M, Alouf, J.E., Geoffroy, C., and Möllby, R. (1981). Membrane-damaging action of alveolysin from Bacillus alvei. *Infect. Immun*. **32,** 1187–1192.

Todd, E.W. (1932). Antigenic streptococcal hemolysin. *J. Exp. Med*. **55,** 267–280.

Todd, E.W. (1934). A comparative serological study of streptolysins derived from human and from animal infections, with notes on pneumococcal hemolysin, tetanolysin, and *staphylococcus* toxin. *J. Pathol. Bacteriol*. **39,** 299–321.

Todd, E.W. (1938). The differentiation of two distinct serological varieties of streptolysin, streptolysin O, and streptolysin S. *J. Pathol. Bacteriol*. **47,** 423–445.

Todd, E.W. (1941). The oxygen-labile hemolysin or toxin of *Clostridium welchii. Brit. J. Exp. Pathology* **22,** 172–178.

Tweten, R.K. (1988). Cloning and expression in *Escherichia coli* of the perfringolysin O (theta toxin) gene from *Clostridium perfringens* and characterization of the gene product. *Infect. Immun*. **56,** 3228–3234.

Tweten, R.K., Harris, R.W. and Sims, P.J. (1991). Isolation of a tryptic fragment from *Clostridium perfringens* toxin that contains sites for membrane binding and self-aggregation. *J. Biol. Chem*. **266,** 12449–12454.

Tweten, R., Parker, M. and Johnson, A. E. (2001). The cholesterol-dependent cytolysins. In: *Pore-Forming Toxins* (ed. F. G. van der Goot), pp. 15–33. Springer, Berlin.

Vanier, G., Segura, M., Friedl, P., Lacouture, S. and Gottschalk, M. (2004). Invasion of the porcine brain microvascular endothelial cells by *Streptococcus suis* serotype 2. *Infect. Immun*. **72,** 1441–1449.

Vázquez-Boland, J.A., Dominguez-Bernal, G., Gonzalez-Zorn, B., Kreft, J. and Goebel, W. (2001). Pathogenicity islands and virulence evolution in *Listeria. Microbes Infect*. **3,** 571–584

Vázquez-Boland, J. A., Dominguez, L., Rodriguez-Ferri, E. F., Fernandez-Garayzabal, J. F. and Suarez, G. (1989). Purification and characterization of two *Listeria ivanovii* cytolysins, a sphingomyelinase C and a thiol-activated toxin (ivanolysin O). *Infect. Immun*. **57,** 3928–3935.

Walker, J.A., Allen, R.L., Falmagne P., Johnson, M.K. and Boulnois, G. (1987). Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae. Infect. Immun*. **55,** 1184–1189.

Watson, K.C. and Kerr, E.J.C. (1974). Sterol requirements for inhibition of streptolysin O activity. *Biochem. J*. **140,** 95–98.

Weld, J.T. (1935). Further studies with serum extracts of hemolytic streptococci. *J. Exp. Med*. **61,** 473–477.

Wheeler, M.W. and Humphreys, E.M (1924). Isolation of B. Botulinus type B from feces by use of blood agar plates in anaerobic jar. *J. Infect. Dis*. **35,** 305–310.

Wuth, O. (1923). Serologische und biochemische Studien das Hämolysin des Fränkelschen Gasbrand-bazillus. *Biochem. Z*. **142,** 19–28.

Yutsudo, T. (1994). Amino acid sequence of cereolysin (derived from nucleotide sequence PID: g600259) *Genbank Accession D* 21270.

Zitzer, A., Westover, E.K., Covey, D.L. and Palmer, M. (2003). Differential interaction of the two cholesterol-dependent, membrane-damaging toxins, streptolysin O and *Vibrio cholerae* cytolysin, with enantiomeric cholesterol. *FEBS Lett*. **553,** 229–231.

CHAPTER

# 37

# Comparative three-dimensional structure of cholesterol-dependent cytolysins

*Galina Polekhina, Susanne C. Feil, Julian Tang, Jamie Rossjohn, Kara Sue Giddings, Rodney K. Tweten, and Michael W. Parker*

## INTRODUCTION

The cholesterol-dependent cytolysins (CDCs) are one of the most widely distributed toxins known. The toxin gene or its product has been identified in numerous species from seven different genera of Gram-positive bacteria, including the *Clostridium*, *Bacillus*, *Streptococcus*, *Listeria*, *Brevibacillus, Paenibacillus,* and most recently the *Arcanobacterium* (Tweten *et al*., 2001). The fact that this gene is so widely distributed among these various pathogenic bacteria suggests that it plays an important role in the pathogenic mechanism of these organisms. For example, listeriolysin O is an essential virulence factor that is responsible for the release of the intracellular pathogen *Listeria monocytogenes* from the phagocytic vacuole (Jones and Portnoy, 1994).

The CDCs exhibit many unique features, including an absolute dependence of their cytolytic activity on the presence of cholesterol in the membrane and also the formation of very large oligomeric pores, more than 150 Å in diameter, on the membranes of cells. These toxins have been shown to be cytolytic to many eukaryotic cell types, although the bulk of the literature has focused on the hemolytic activity of these toxins. The crystal structures of two CDCs have been solved, and experimental approaches combining molecular biology techniques and various biophysical analyses have helped uncover fundamental features by which these toxins assemble and insert into the membrane. Several excellent reviews have been published on these toxins (Alouf and Geoffroy, 1991; Alouf, 2000; Gilbert *et al*., 2000; Palmer, 2001; Heuck *et al*., 2001; Tweten *et al*., 2001; Gilbert, 2002), so this review will focus on recent advances in structural studies of CDC toxins. Biological aspects of the toxins discussed in this chapter are covered in other chapters of Section III, particularly in Chapter 38 "Perfringolysin O and intermedilysin: mechanisms of pore formation by the cholesterol-dependent cytolysins."

## CDC PRIMARY STRUCTURES

There are more than 20 members of the CDC family so far identified, and there exists a high degree of sequence similarity (40–80%) among them, suggesting they all have similar activities and 3D structures. The primary structures of streptolysin O (SLO), pneumolysin (PLY), and perfringolysin O (PLO) were revealed in the late 1980s by DNA sequence analyses of their genes (Kehoe *et al*., 1987; Tweten *et al*., 1988; Walker *et al*., 1987). These studies showed that all three toxins exhibited a significant level of identity in their primary structures (Figure 37.1), and that each contained a single cysteine residue in a highly conserved region near the C-terminal end of each protein. It was this cysteine, upon modification with thiol-specific reagents, that was responsible for the loss in cytolytic activity of these toxins (Iwamoto *et al*.,

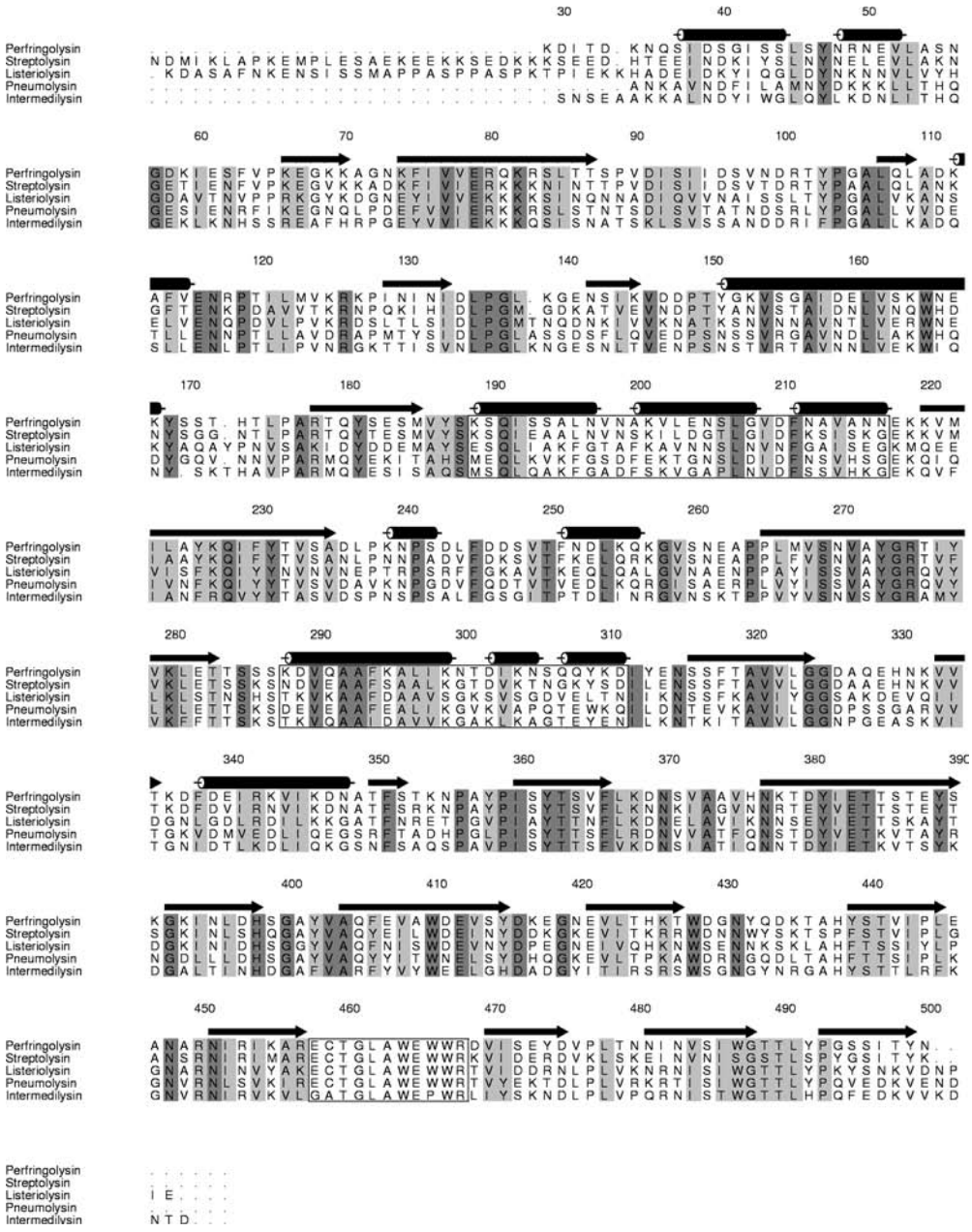



**FIGURE 37.1** Sequence alignment of various cholesterol-dependent cytolysins. Numbering refers to the PFO sequence; strictly conserved regions are shaded in dark gray, and highly conserved regions are shaded in light gray. The secondary structure of PFO, as seen in the crystal structure, is shown above the alignments (helices as cylinders and strands as arrows). The TMH1, TMH2, and undecapeptide regions are shown boxed, in that order, from the N-terminus.

1987). However, this single cysteine residue was shown not to be required for the *in vitro* cytolytic activity of SLO and PLY (Saunders *et al*., 1989; Pinkney *et al*., 1989), and more recently for PFO (Shepard *et al*., 1998). Substitution of the cysteine with alanine by *in vitro* mutagenesis yielded a toxin molecule that was similar in activity to that of the cysteine-containing wild-type. However, substitution of the cysteine with serine or glycine caused a significant decrease in the cytolytic activity of SLO and PLY (Saunders *et al*., 1989; Pinkney *et al*., 1989). Therefore, even though the sulfhydryl group is not required for the *in vitro* cytolytic activity of these toxins, the cysteine residue apparently occupied a site within the toxin structure that is critical to the function of the CDC. Why the cysteine has been retained at this position in these toxins, when alanine would function equally well and is not susceptible to oxidation, is not clear. Of the 11 sequenced CDCs, only pyolysin (PLO) from *Arcanobacterium pyogenes* and intermedilysin (ILY) from *Streptococcus intermedius* (Figure 37.1) have an alanine substituted for the cysteine. Although it is clear that the sulfhydryl is not required for cytolytic activity, it is not known if it has some as yet undefined role *in vivo*.

A cysteine-containing, highly conserved undecapeptide sequence, ECTGLAWEWWR, is present in eight of the 11 sequenced CDCs (Figure 37.1). The remaining three toxins exhibit various substitutions in this region, some of which are conservative and others of which are not. In addition to containing the cysteine residue, this region also contains a conspicuously large number of tryptophan residues: 10 of the 11 sequenced toxins contain three tryptophans in the undecapeptide. The undecapeptide sequence of seeligeriolysin from *Listeria seeligleri* has a single residue change in this sequence in which a phenylalanine is substituted for an alanine. However, the more recently discovered and sequenced CDCs, PLO (Billington *et al*., 1997) and ILY (Nagamune *et al*., 1996; Nagamune *et al*., 2000), exhibit significant differences in this region (Figure 37.1). Both have an alanine substituted for the cysteine residue, thus making them resistant to inactivation due to oxidation of the sulfhydryl. These two toxins also have significant differences in the last 4–5 residues of the undecapeptide. PLO has a conservative change of Glu to Asp (Asp498 of PLO), but also contains an insertion of a proline between Asp498 and Trp500. ILY also exhibits the same aspartate to glutamate and cysteine to alanine changes as PLO, but instead of inserting a proline, a proline has been substituted at position 494, a position where tryptophan would normally be found (Figure 37.1). Therefore, ILY only contains two tryptophans in this region, whereas all of the other toxins have three tryptophans. The role of the highly conserved undecapeptide in the cytolytic mechanism has not yet been completely clarified.

There are many additional differences in the primary structures of these toxins, none of which has been linked to a unique function of a particular CDC. However, one difference is worth noting and is unique to the structure of SLO, which is produced by *Streptococcus pyogenes*. When the SLO amino acid sequence is aligned with the primary structures of the other CDCs, an additional 70–75 amino acids are present at its amino terminus. The additional residues in SLO are located between the predicted signal peptide and the region of SLO that aligns with the approximate amino termini of the secreted forms of the other toxins. This additional sequence does not align with any of the other CDCs and does not exhibit any significant sequence similarity with any other protein. It has been shown that SLO can be nicked with the cysteine proteinase of *S. pyogenes* between residues Lys 77 and Leu 78 (Gerlach *et al*., 1993). This cleavage removes 46 amino acids from the secreted SLO and yields a 55.5 kDa protein. Both the uncleaved and cleaved forms are hemolytically active. Although this cleavage removes a significant portion of the extra sequence, there still remains an extra 26 residues on the small form of SLO, which does not exhibit any sequence similarity with the other CDCs. If the amino terminal region has a function specific for SLO, it has yet to be demonstrated.

## PFO—AN ARCHETYPICAL CDC

*Clostridium perfringens* is a normal inhibitant of the gastrointestinal tract of humans and animals, as well as being commonly found in soil. It causes human gas gangrene and food poisoning, as well as several enterotoxemic diseases in animals. The pathogenesis of gas gangrene (or clostridial myonecrosis) primarily involves the invasion of traumatic wounds or deoxygenated tissues by the Gram-positive bacterium. *C. perfringens* releases numerous virulence factors, including two lethal toxins, alpha-toxin and PFO, which are thought to be the major virulence factors of the bacterium. PFO, or theta-toxin, induces tissue destruction and anti-inflammatory responses and acts synergistically with alpha-toxin in gangrenous lesions (Rood, 1998). PFO is produced by all strains of *C. perfringens*. Its gene encodes a polypeptide that consists of a 27 amino acid signal peptide followed by 500 amino acids of the mature protein.

## CRYSTAL STRUCTURE OF PFO

The crystal structure of PFO was determined in 1997 (Rossjohn *et al*., 1997). The structure was originally determined to a resolution of 2.7 Å, but has since been extended to a resolution of 2.2 Å (Rossjohn, J., Polekhina, G., Feil, S.C. McKinstry, W.J., Tweten, R.K. and Parker, M.W., unpublished results). The PFO molecule is a very elongated molecule with its long axis measuring approximately 115 Å (Figure 37.2). A notable feature of the secondary structure is that it is very rich in beta-sheet. Although the molecule did not closely resemble any other molecule for which a crystal structure was known, its shape and secondary structure content were reminiscent of a number of other toxins including aerolysin (Parker *et al*., 1994), *Staphyloccus* α-hemolysin (Song *et al*., 1996), the protective antigen of anthrax toxin (Petosa *et al*., 1997), and LukF (Olson *et al*., 1999; Pédelacq *et al*., 1999).

The crystal structure demonstrated that PFO is built from four domains: the N-terminal domain or domain 1 (residues 37–53, 90–178, 229–274, 350–373), domain 2 (residues 54–89, 374–390), domain 3 (residues 179–228, 275–349), and the C-terminal domain or domain 4

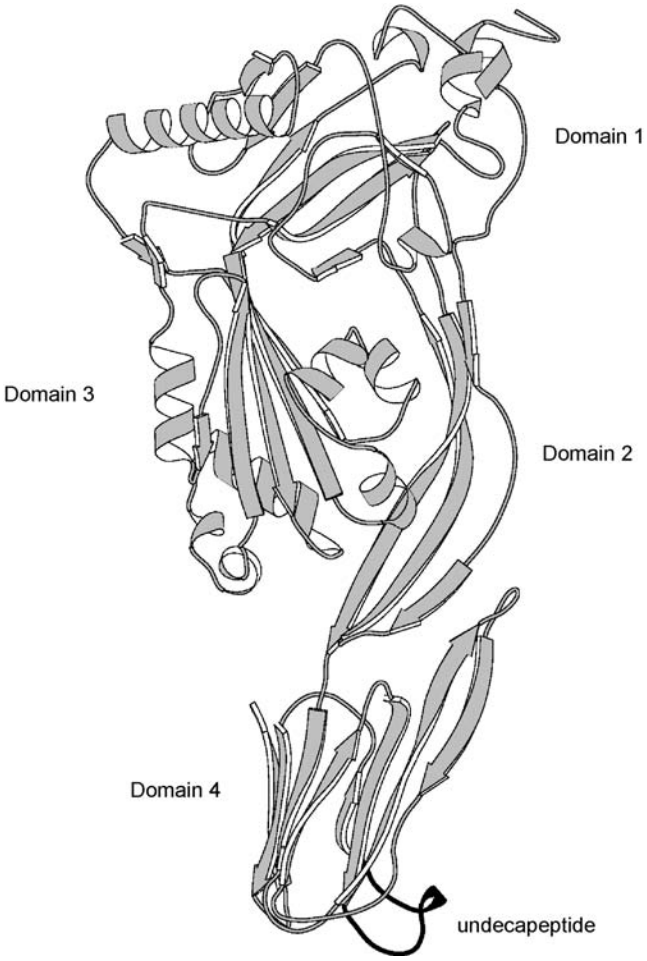



**FIGURE 37.2** Crystal structure of PFO. A ribbon representation indicating the location of domains and the undecapeptide motif (in dark shade). The figure was drawn using MOLSCRIPT (Kraulis, 1991).

(residues 391–500) (Figure 37.2). Domain 1, located at one end of the molecule, adopts an alpha-beta topology with a long helix packing against a core of anti-parallel beta-sheet. Domain 2 consists of a single layer of anti-parallel beta-sheet connecting one end of the molecule to the other. Domain 3 also adopts alpha-beta topology with a core anti-parallel beta-sheet surrounded by helical layers on both sides. Domain 4 adopts a beta-sandwich topology, a common fold found in a variety of proteins.

Two of the domains, domains 3 and 4, exhibited unusual features. The core beta-sheet that runs through domains 1 and 3 has a highly pronounced curvature at the domain interface. The packing of domain 3 onto the rest of the protein is far from complementary and involves predominantly polar contacts. (Normally, domain interfaces have very complementary surfaces and regions of hydrophobic contacts). These two features of domain 3 suggested the possibility that it could readily flex away from domain 2 so as to relieve the energetically unfavorable stress at the domain 1–3 interface.

Domain 4 was of particular interest because it houses the undecapeptide sequence, which had previously been implicated in cholesterol and membrane binding. This sequence was found to form an extended loop with a single turn of helix at the tip of the molecule. The loop curls back into one of the beta-sheets so that the tip of the loop defined by Trp 464 is nestled into the hydrophobic core of a number of long surface side chains. This immediately suggested that this site might be the cholesterol-binding site with the tryptophan side chain mimicking how a cholesterol molecule would bind if the loop was displaced. The undecapeptide motif itself consists of mostly hydrophobic residues, so its displacement would generate a hydrophobic "dagger" that would be capable of inserting into a membrane. Domain 4 is connected to the rest of the protein through a single linking peptide, suggesting that it too could be quite mobile in solution.

## STRUCTURE/FUNCTION STUDIES OF PFO

A major advance in understanding the mechanism of CDC membrane insertion came with the discovery that domain 3 harbored regions that formed the transmembrane (TM) β-barrel. The studies of Shepard *et al*. and Shatursky *et al*. revealed that a series of small helices on either side of a central β-sheet could unfurl into β-hairpins, TMH1 and TMH2, that were capable of spanning membrane bilayers (Figure 37.3). Such conformational changes were unprecedented among toxins and hence represented a new paradigm for how pore-forming proteins generated a membrane-spanning domain.

PFO must interact with membranes as a prerequisite for the insertion of the domain 3 TM hairpins that form the wall of the transmembrane β-barrel (Heuck *et al*., 2000). The conserved undecapeptide and three other short hydrophobic loops, all located at the tip of domain 4 of PFO, penetrate the surface of the membrane and anchor the molecule for the subsequent conformational changes during pore formation (Heuck *et al*., 2000; Nakamura *et al*., 1995; Ramachandran *et al*., 2002). Domains 3 and 4 are conformationally coupled, although they are not in direct physical contact. Mutations in domain 3 can affect the rate at which domain 4 interacts with the membrane, even though the domain 4 undecapeptide interacts with the membrane prior to the insertion of the domain 3 TMHs

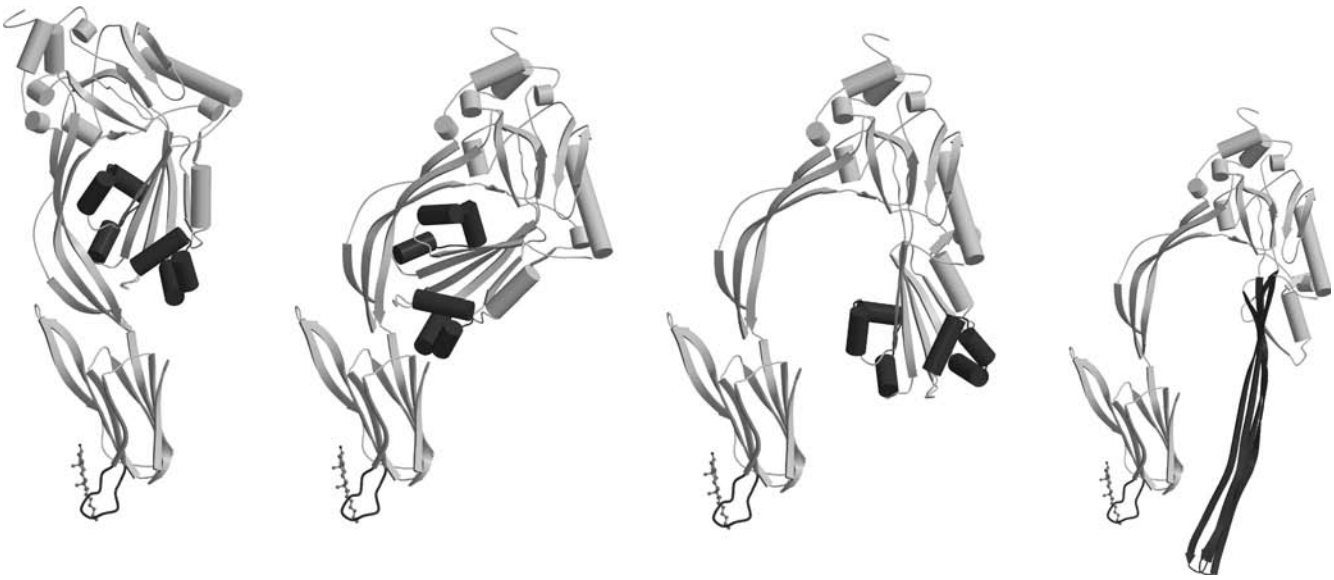


**FIGURE 37.3** A schematic model of stages of CDC insertion into membranes. From left to right: Ribbon representation of the PFO monomer as seen in the crystal structure. This is the structure thought to exist in solution. The TMH1 and TMH2 are highlighted in dark shade, and the undecapeptide loop is shown sprung out with a molecule of cholesterol binding to it. Next, domain 3 starts to rotate away from the body of the molecule, and the TMH regions are released simultaneously to form extended beta hairpins. It is likely that domain 4 curls into the body of the molecule, allowing the TMH regions to fully puncture the membrane so as to form a beta-barrel with the other monomers of the oligomer. Only one monomer is shown for clarity. This figure was produced using MOLSCRIPT (Kraulis, 1991).

(Palmer *et al*., 1998; Abdel Ghani *et al*., 1999; Heuck *et al*., 2000; Hotze *et al*., 2001; Ramachandran *et al*., 2002). The cytolytic activity of PFO is highly sensitive to changes in the undecapeptide motif, especially mutations of the conserved tryptophans or chemical modification of the thiol of the conserved cysteine, which inhibit membrane binding (Nakamura *et al*., 1995; Sekino-Suzuki *et al*., 1996, Alouf, 2000; Iwamoto *et al*., 1987). However, some undecapeptide mutations or modifications appear to affect the global structure of PFO, thus suggesting that its conformation is important to the proper function of the toxin.

A detailed model of the initial stages of membrane insertion by CDCs is now starting to emerge (Figure 37.3). In the first step, the toxin binds to cell surfaces via domain 4. The undecapeptide loop flips out from this domain to form a membrane-penetrating hydrophobic dagger that anchors the protein to the membrane. Many studies suggest that cholesterol acts as a receptor in this step, triggering the conformational change, although recent data suggests the role of cholesterol in this step is toxin dependent (Giddings *et al*., 2003). The partial insertion of domain 4 would bring domain 3 closer to the membrane surface and also would make the toxin oligomerization-competent (Abdel Ghani *et al*., 1999). A recent study suggests membrane binding is associated with conformational changes in domain 3 (Abdel Ghani *et al*., 1999). A prepore complex assembles on the membrane surface by lateral diffusion of membrane-bound monomers (Shepard *et al*., 2000; Hotze *et al*., 2001; Hotze *et al*., 2002). Electron microscopy studies of the CDC, pneumolysin, show that the prepore complex is bound to the membrane via the base of domain 4 where the undecapeptide loop resides (Gilbert *et al*., 1999). The TMH regions in domain 3 are kept unfurled at this stage to prevent premature membrane insertion of isolated monomers (Shepard *et al*., 2000; Heuck *et al*., 2000; Hotze *et al*., 2001). The formation of the oligomer triggers a further conformational change in domain 4, causing a rotation of domain 4 away from the long axis of the molecule and a concerted rotation of domain 3 as described previously. The rotation of domain 3 away from the molecule in the oligomer has been directly observed by electron microscopy (Gilbert *et al*., 1999). These rotations are of the order of 35° to 45° for domain 4 and would be sufficient to break all contacts between domains 2 and 3 and allow the concerted unfurling of the TMH regions to form β-hairpins (Hotze *et al*., 2001). Electron microscopy studies of pneumolysin oligomers support our suggestion that domain 3 swings out from the body of the molecule to adopt a disordered conformation and that the rotation of domain 3 is coupled to domain 4 rotations (Gilbert *et al*., 1999). It has been argued that a concerted insertion of the β-hairpins from all monomers of the oligomer then occurs based

on energetic considerations (Heuck *et al*., 2001), and this hypothesis is supported by recent experimental data (Hotze *et al*., 2002). Our modeling of the oligomer suggests that domain 4 must curl into the body of the molecule so that the extended β-hairpins can penetrate the bilayer. Very recently, a vertical collapse of about 40 Å was observed by atomic force microscopy between the prepore and pore states of PFO. The data is consistent with domain 2 collapsing into a more globular domain, rather than the elongated domain seen in the crystal structure of the water-soluble oligomer (Czajkowsky *et al*., 2004).

## NEW CRYSTAL STRUCTURE OF PFO

The structure of PFO has recently been solved in another crystal form at 3 Å resolution (Rossjohn, J., Polekhina, G., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W., unpublished results). These crystals were grown using butanol as the precipitant, and they contain two molecules per asymmetric unit. The two molecules pack in a head-to-tail arrangement and are essentially identical to each other, with a root-mean-square (r.m.s) deviation of 0.6 Å over all Calpha atoms. They also superpose well with the previously published structure, with an overall r.m.s deviation of 1.0 Å over all Calpha atoms. The conformation of the undecapeptide motif is also very similar, as is the conformation of the TMH1 and TMH2 helical springs, demonstrating that the observed conformations of these critical regions are not artefacts of the cystallization process.

The overall temperature factor, a measure of the order of the molecule, was mapped onto the structures for both the crystal forms. The β-sandwich of domain 4 and the 5-stranded sheet of domain 3 represent the most ordered part of the molecule in the published crystal form. The top and neck of the molecule appears largely mobile, with the outer region of domain 2 being very mobile. The new crystal form shows an overall increased mobility over the entire backbone, despite showing no large-scale structural differences with respect to the wild-type structure. Domain 4 and the β-sheet of domain 3 represent the most ordered regions of the molecule as well.

## MOLECULAR BASIS OF THIOL ACTIVATION

CDCs were initially termed the thiol-activated cytolysins because thiol-reagents such as N-ethyl maleimide and mercury salts (Alouf and Geoffroy, 1991) inhibited cytolytic activity, whereas thiol-reducing reagents reversed these effects. This led to the concept of the "essential thiol." However, it was subsequently shown that the cysteine is not essential *in vivo* (see above). Each CDC contains just one cysteine throughout the sequence, with the exception of ivanolysin, which contains two cysteines. This solitary cysteine is located in the undecapeptide motif, and thus the effects of the thiol-reagents must be directed to the cysteine within the motif. The structural basis of inactivation by these thiol reagents is unknown.

During the course of the structure determination of PFO (Rossjohn *et al*., 1997), two mercury derivatives, mercury acetate (HgOAc) and *p*-chloromercuri-benzene sulfonate (PCMBS), were used. The PCMBS and HgOAc data sets extend to 2.7 Å and 2.9 Å, respectively. To address the structural basis of toxin inactivation, the two mercury-modified forms of PFO have been refined (Rossjohn, J., Polekhina, G., Feil, S.C. McKinstry, W.J., Tweten, R.K. and Parker, M.W., unpublished results). The electron density for both models, in particular the region around the undecapeptide motif, is well defined. The structures of PCMBS-PFO and HgOAc-PFO superpose well with respect to the 2.2 Å resolution wild-type structure. Somewhat surprisingly, the region around the undecapeptide in both metal complexes is virtually identical to the wild-type (Figure 37.4). The chemical modification of Cys 459 can be seen not to disrupt appreciably the conformation of the undecapeptide motif, implying that these thiol reagents must inactivate PFO via another mechanism. One possibility is that cysteine modification affects the flexibility of the undecapeptide loop, which has been hypothesized to flip out from the body of the molecule on binding cholesterol to form a membrane-penetrating hydrophobic dagger (Rossjohn *et al*., 1997; Ramachandran *et al*., 2002; and see below).

## INTERMEDILYSIN—AN ATYPICAL CDC

*S. intermedius* secretes the CDC ILY (Macey *et al*., 2001), and ILY appears to be a major virulence factor of *S. intermedius*, as it is expressed up to 10-fold more in abscesses (Nagamune *et al*., 2000). ILY exhibits three features that distinguish it from most other CDCs. First, it has a variant undecapeptide sequence (GATGLAWEPWR) (Figure 37.1). The normally conserved cysteine of the CDC undecapeptide is replaced by an alanine residue, resulting in an oxidation-resistant toxin (Nagamune *et al*., 1996), and a proline

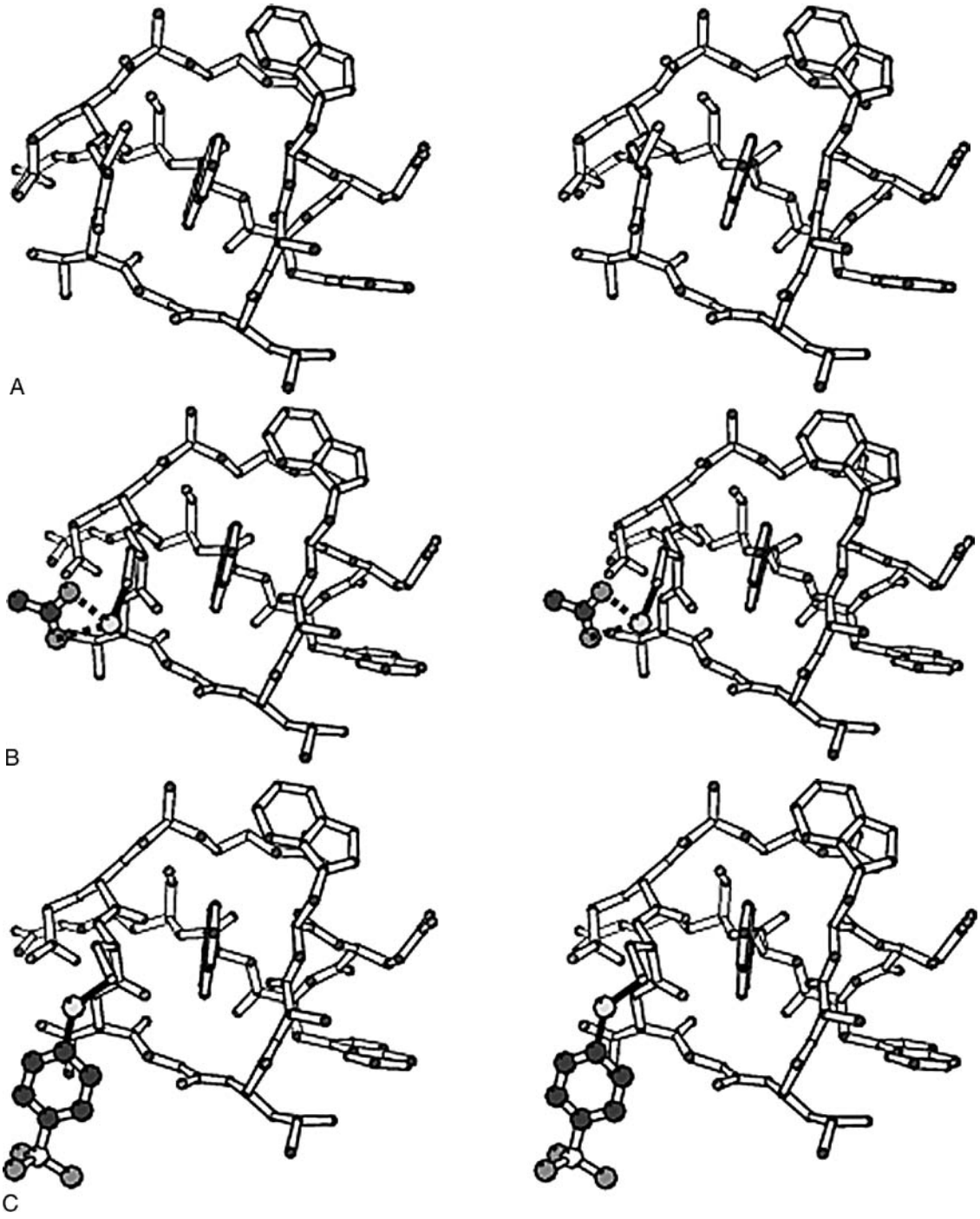



**FIGURE 37.4** Stereoview of the mercury complex structures in the vicinity of the undecapeptide loop in domain 4. The sulfur atom of the unique thiol of residue Cys 459 packs against the aromatic ring of Trp 467. (A) Wild-type. (B) Mercury acetate complex. (C) *p*-Chloromercuribenzene sulfonate complex.

residue is substituted for the second conserved tryptophan. Second, ILY is specific for human cells (Nagamune *et al.*, 1996; Macey *et al.*, 2001). Nagamune and coworkers (1996) showed that animal erythrocytes from nine species are not susceptible to ILY and non-human primate erythrocytes are a hundred-fold less susceptible to ILY than human erythrocytes. Third, free cholesterol is only weakly inhibitory. Although the presence of membrane cholesterol is still essential to the activity of ILY, it does not appear

to function as a receptor as it does for PFO (Giddings *et al*., 2003). Giddings and coworkers (2003) showed that depletion of approximately 90% of the membrane cholesterol did not affect ILY binding or that of the related CDC, SLO, to human erythrocytes, but it did completely abrogate the hemolytic activity of both toxins. It was determined by fluorescence spectroscopy that cholesterol depletion prevented the prepore to pore conversion of ILY and SLO. Therefore, cholesterol was necessary for ILY membrane insertion, but not its membrane binding.

The unique aspects of the ILY mechanism, its variant undecapeptide and human cell specificity, prompted us to investigate whether the two aspects were linked. We have pursued a detailed structural investigation of ILY with the aim of understanding how these distinguishing features impact on its interaction with cells.

## CRYSTAL STRUCTURE OF ILY

The crystal structure of ILY has been determined to a resolution of 2.6 Å (Polekhina *et al*., 2004; Polekhina *et al*., 2005). Overall, ILY adopts a similar structure to PFO (see next section), so only a brief overview of the structure will be presented here. It is an elongated boomerang-shaped molecule of dimensions 50 Å × 58 Å × 114 Å (Figure 37.5). The molecule is rich in β-sheet with 40% sheet made up of 23 β-strands contributing to four sheets. One strand (residues 399 to 425) spans about two-thirds of the molecule and measures 100 Å in length. There are 15 helices, varying in length between 4 and 21 residues.

The molecule is comprised of four discontinuous domains. Domain 1 (residues 56 to 79, 116 to 205, 256 to 301, 377 to 400) has an α/β structure with a core of six-stranded β-sheet. Domain 2 (residues 80 to 115, 401 to 417) consists of a three-stranded β-sheet. Domain 3 (residues 206 to 255, 302 to 376) is comprised of an α/β/α three-layer structure. The interface of domains 2 and 3, covering a surface area of 592 Å$^2$, is constructed from the packing of two helices against the β-sheet of domain 2 and consists of four potential hydrogen-bonding interactions and 17 van der Waals interactions. Domain 2 is covalently connected to domain 4 through a single connection. Domain 4 (residues 418 to 528) is folded into a compact β-sandwich consisting of four- and five-stranded sheets. The interface between domains 2 and 4, covering 242 Å$^2$, consists of one salt link (between Arg 92 and Glu 448), three hydrogen-bonding interactions, and 10 van der Waals interactions. In addition, there are numerous

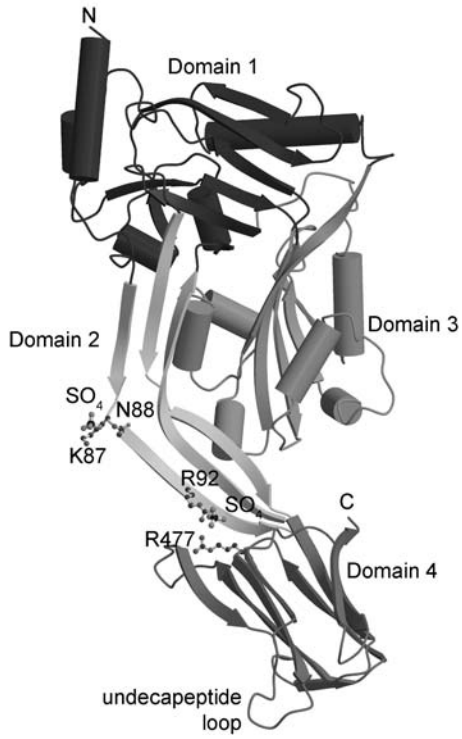



**FIGURE 37.5** Crystal structure of ILY. A ribbon representation indicating the location of domains and the undecapeptide loop. The figure was drawn using MOLSCRIPT.

sulfate ion-mediated contacts between the two domains (see below).

ILY crystallized with two molecules in the asymmetric unit. The two molecules superimpose with a r.m.s. deviation of 2.2 Å on Calpha positions. This large deviation is due to a relative 10° rotation of domain 4 with respect to the rest of the molecule, so that the tips of domain 4 are nearly 16 Å apart when domain 1 of each molecule is superimposed. Superposition of domains 1 to 3 yields a r.m.s. deviation of 0.7 Å and superposition of domain 4 yields a deviation of 0.6 Å. Hence, aside from the rotation of domain 4, the two molecules are very similar.

Sulfate ions were a vital ingredient for crystallization of ILY (Polekhina *et al*., 2004). Four sulfate ions were identified in the asymmetric unit and were found to bind to the same two sites on each of the two protein molecules. This suggests the sulfate binding sites may be more than just an artifact of the crystallization process. One sulfate ion is bound to Arg 92 and Arg 477 and hence bridges domains 2 and 4 (Figure 37.5). Another sulfate is bound in an unusual manner as it is coordinated by two consecutive residues Lys 87 and Asn 88 (through both side chain and main chain atoms), both from domain 2, as well as Arg 477 from

the opposing molecule (Figure 37.5). It is conceivable that the sulfate-binding sites might represent binding pockets for the putative human protein receptor.

## STRUCTURAL COMPARISON OF ILY AND PFO

The most similar structure to ILY in the Protein Data Bank (http://www.rscb.org/pdb) is PFO (Rossjohn *et al*., 1997), the only other CDC for which a three-dimensional atomic structure has been determined. The two proteins have an overall pairwise sequence identity of 40% (197 out of a total of 532 residues) that is distributed relatively evenly over the entire sequence of each molecule. Both proteins share the same number of domains, and each domain shares the same overall topology. The most striking difference between the two molecules is the highly bent shape of ILY compared to PFO, with domain 4 rotated between 20˚ and 30˚ away from the main axis of the ILY molecule (Figure 37.6). (The rotation is either 20˚ or 30˚, depending on which ILY molecule in the asymmetric unit is used in the superposition.)

Domain 4 is the most similar domain between the two molecules with an r.m.s. deviation on 91 equivalenced Calpha atoms of 0.8 Å. The most significant difference in this domain is the conformation of the undecapeptide loop that adopts an extended conformation in ILY, but is curled up against the β-sheet in PFO (Figure 37.6). The extended conformation does not appear to be an artifact of the crystallization, as the loop of one of the two ILY molecules in the asymmetric unit is not involved in crystal contacts, and the loops of both molecules superimpose almost exactly. There is also a movement of a hairpin loop by almost 10 Å at the other end of this domain due to differences in domain 2 (Figure 37.6). Another notable difference is the distribution of electrostatic charge on the surface of domain 4 of each toxin. ILY is predominantly positively charged, whereas PFO is negatively charged with differences particularly pronounced in the region of the undecapeptide motif and at the C-terminus.

A comparison of the domain 4 primary structures of ILY and PFO does not provide any obvious candidate regions of domain 4 that may be involved in cellular specificity of ILY (Figure 37.1). About 60% of the residues are not conserved between the two domains, and these differences are distributed comparatively evenly throughout domain 4. A surface analysis over the domain 4 region of the crystal structures is also not illuminating. There are no compelling patches of unique or unusual sequence on the surface of this domain that would suggest a receptor-binding region.

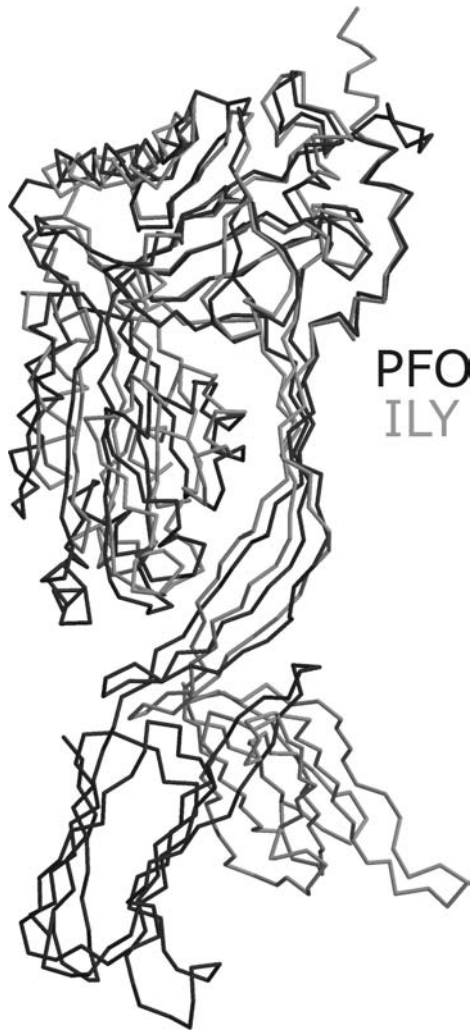



**FIGURE 37.6** Superposition of ILY and PFO. Calpha traces of ILY (light gray) and PFO (black), based on a domain 1 superposition.

Domain 1 is the next closest with 1.2 Å r.m.s. deviation on 145 equivalenced Calpha atoms (Figure 37.6). The most significant differences in this domain are the positions of the two helices seen in PFO with the longer helix (residues 178 to 195) shifted by about 3.5 Å and the shorter one (residues 278 to 284) by 2.8 Å. There is also an additional short helix (residues 122 to 127) in ILY not seen in PFO. The N-terminal helix (residues 56 to 70) in ILY is longer and more ordered than in PFO and is also shifted by about 3 Å compared to PFO.

Domain 3 with an r.m.s. deviation of 3.1 Å (over 130 equivalenced Calpha atoms) does not superimpose well because, although the core β-sheet is similar (r.m.s. deviation of 1.5 Å over 38 equivalenced atoms), the helices packing on either side of the sheet adopt different positions relative to the sheet (Figure 37.6).

In particular, the helices on the surface of the ILY molecule (TMH2) are not as closely nestled to the sheet as they are in PFO (with shifts of up to 7 Å), and they exhibit high temperature factors. The helices at the interface between domains 3 and 2 (TMH1) also shift by up to 3 Å compared to PFO. If the two molecules are superimposed using domain 1 only, domain 3 is shifted by about 2 Å in ILY compared to PFO (Figure 37.6). Domain 2 does not superimpose well due to a twist in the β-sheet in the middle of the domain, causing a r.m.s. deviation of 2.9 Å over 51 equivalenced Calpha atoms (Figure 37.6). This deviation is responsible for the pronounced kink in the shape of the molecule compared to PFO.

The solution of the ILY structure demonstrates that the basic features of the CDC 3D structure appear to be conserved among the CDCs. A phylogenetic tree generated by the alignment of the known CDC primary structures (15 in all), using the nearest-neighbor method of Saitou and Nei (1987), shows that PFO and ILY are two of the most distantly related CDCs (data not shown), yet they exhibit similar 3D structures. Therefore, it is likely that all CDCs will exhibit 3D structures similar to that of PFO and ILY.

## CHOLESTEROL-BINDING SITES

Previous work has implicated cholesterol as the receptor for the CDC family, where it is thought to act by concentrating the toxin in cholesterol-rich domains of the target cell membrane to promote oligomerization (Alouf and Geoffroy, 1991). We have previously hypothesized that cholesterol could bind to PFO by displacing the undecapeptide loop and binding to one face of the β-sheet in domain 4 (Rossjohn *et al*., 1997). A striking feature of the ILY structure is the extended conformation of the undecapeptide loop (Figure 37.6), a conformation predicted for PFO when it binds cholesterol. We have computationally docked a cholesterol molecule into the ILY structure and found no preferred clustering of cholesterol molecules into the putative binding site (Polekhina *et al.*, 2005). The conformation of the undecapeptide loop in ILY provided a basis for modeling the equivalent loop, in its sprung-out form, in PFO. We then docked cholesterol into the modified PFO structure and found that the majority of docks yielded a close clustering of cholesterol molecules with the same orientation and position in the putative binding pocket. The cholesterol molecule was found to stack against the aromatic side chains of Trp 466 and 467 and the 3β-hydroxy moiety, forming a hydrogen bond to Glu 407 of PFO. A comparison of the key residues involved in interacting with cholesterol in PFO with the equivalent residues in ILY showed that some are not conserved. Glu 407 (PFO) is replaced by Tyr 434 in ILY, and this residue does not appear capable of providing a hydrogen bond to the 3β-hydroxyl of cholesterol. Trp466 (PFO) is substituted by Pro493 in ILY; the latter residue is likely responsible for the extended conformation of the undecapeptide observed in ILY and also disrupts the aromatic stacking interaction between cholesterol and the tryptophans suggested in the PFO model.

In summary, PFO possesses a plausible binding site in domain 4 for a cholesterol molecule if it is assumed that the undecapeptide adopts an extended conformation, as observed in the crystal structure of ILY. Surprisingly, ILY is not predicted to possess such a site. We hypothesize that ILY does not need a cholesterol-binding site because its undecapeptide is already "sprung out." These suggestions are in keeping with earlier work that has shown that free cholesterol is only weakly inhibitory for ILY cytolytic activity in contrast to PFO (Nagamune *et al*., 1996). Nevertheless, cholesterol does play a role in the insertion process of ILY (Giddings *et al*., 2003), indicating that cholesterol may play multiple roles in CDC activity.

## CONCLUSION

The crystal structure of PFO has proved a valuable basis for numerous structure-function studies that have revealed many details of the membrane insertion process (Shepard *et al*., 1998; Shatursky *et al*., 1999; Heuck *et al*., 2000; Shepard *et al*., 2000; Hotze *et al*., 2001; Hotze *et al*., 2002; Ramachandran *et al*., 2002; Heuck *et al*., 2003; Czajkowsky *et al*., 2004; Ramachandran *et al*., 2004). The structure of ILY, an atypical CDC, demonstrates that the overall protein fold first seen in PFO is almost certainly maintained in all members of the family. Key differences include different orientations of domain 4 with respect to the rest of the protein and different conformations of the undecapeptide loop, presumably in response to sequence differences in the region. Further structural studies are required to illuminate the role of cholesterol in CDC activity and the molecular detail of the CDC oligomer.

## ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia (NHMRC) to Michael Parker and the National

Institutes of Health (NIAID, AI37657) to Rodney Tweten. Galina Polekhina is supported by a NHMRC RD Wright Research Fellowship, Jamie Rossjohn is a Wellcome Trust Senior Research Fellow, and Michael W. Parker is a NHMRC Senior Principal Research Fellow.

## REFERENCES

Abdel Ghani, E.M., Weis, S., Walev, I., Kehoe, M., Bhakdi, S. and Palmer, M. (1999). Streptolysin O: inhibition of the conformational change during membrane binding of the monomer prevents oligomerization and pore formation. *Biochemistry* **38,** 15204–15211.

Alouf, J.E. and Geoffroy, C. (1991). The family of the antigenically-related, cholesterol-binding ("sulphydryl-activated") cytolytic toxins. In: *Sourcebook of Bacterial Toxins* (eds. J.E. Alouf, and J.J. Freer), pp. 147–186. Academic Press, London.

Alouf, J. E. (2000). Cholesterol-binding cytolytic protein toxins. *Int. J. Med. Microbiol*. **290,** 351–356.

Billington, S.J., Jost, B.H., Cuevas, W.A., Bright, K.R. and Songer, J.G. (1997). The *Arcanobacterium* (*Actinomyces*) hemolysin, pyolysin, is a novel member of the thiol-activated cytolysin family. *J. Bacteriol.* **179,** 6100–6106.

Czajkowsky, D.M., Hotze, E.M., Shao, Z. and Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane beta-hairpins to the membrane. *EMBO J.* **23,** 3206–3215.

Gerlach, D., Kohler, W., Gunther, E. and Mann, K. (1993). Purification and characterization of streptolysin O secreted by *Streptococcus equisimilis* (group C). *Infect. Immun*. **61,** 2727–2731.

Giddings, K.S., A.E. Johnson, A.E. and Tweten, R.K. (2003). Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *Proc. Natl. Acad. Sci. USA* **100,** 11315–11320.

Gilbert, R.J.C., Jiménez, J.L., Chen, S., Tickle, I.J., Rossjohn, J., Parker, M.W., Andrew, P.W. and Saibil, H.R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*. *Cell* **97,** 647–655.

Gilbert, R.J.C., Jiménez, J.L., Chen, S., Andrew, P.W. and Saibil, H.R. (2000). Structural basis of pore formation by cholesterol-binding toxins. *Int. J. Med. Microbiol*. **290,** 389–394.

Gilbert, R.J.C. (2002). Pore-forming toxins. *Cell. Mol. Life Sci*. **59,** 832–844.

Heuck, A.P., Hotze, E.M., Tweten, R.K. and Johnson, A.E. (2000). Mechanism of membrane insertion of a multimeric beta-barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. *Mol. Cell* **6,** 1233–1242.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2001) β-barrel pore-forming toxins: intriguing dimorphic proteins. *Biochemistry* **40,** 9065–9073.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2003). Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J. Biol. Chem*. **278,** 31218–31225.

Hotze, E.M., Wilson-Kubalek, E.M., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (2001). Arresting pore formation of a cholesterol-dependent cytolysin by disulfide trapping synchronizes the insertion of the transmembrane beta-sheet from a prepore intermediate. *J. Biol. Chem*. **276,** 8261–8268.

Hotze, E.M., Heuck, A.P., Czajkowsky, D.M., Shao, Z., Johnson, A.E. and Tweten, R.K. (2002). Monomer-monomer interactions drive the prepore to pore conversion of a beta barrel-forming, cholesterol-dependent cytolysin. *J. Biol. Chem*. **277,** 11597–11605.

Iwamoto, M., Ohno-Iwashita, Y. and Ando, S. (1987). Role of the essential thiol group in the thiol-activated cytolysin from *Clostridium perfringens*. *Eur. J. Biochem*. **167,** 425–430.

Jones, S. and Portnoy, D.A. (1994). Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O instead of listeriolysin O. *Infect. Immun*. **62,** 5608–5613.

Kehoe, M.A., Miller, L., Walker, J.A. and Boulnois, G.J. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. *Infect. Immun*. **55,** 3228–3232.

Kraulis, P. (1991). MOLSCRIPT: a program to produce both detailed and schematic plots of proteins. *J. Appl. Crystallogr*. **24,** 946–950.

Macey, M.G., Whiley, R.A., Miller, L. and Nagamune, H. (2001). Effect on polymorphonuclear cell function of a human-specific cytotoxin, intermedilysin, expressed by *Streptococcus intermedius*. *Infect. Immun*. **69,** 6102–6109.

Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R.A., Tsuji, A. and Matsuda, Y. (1996). Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect. Immun*. **64,** 3093–3100.

Nagamune, H., Whiley, R.A., Goto, T., Inai, Y., Maeda, T., Hardie, J.M. and Kourai, H. (2000). Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with *Streptococcus intermedius*. *J. Clin. Microbiol*. **38,** 220–226.

Nakamura, M., Sekino, N., Iwamoto, M. and Ohno-Iwashita, Y. (1995). Interaction of θ-toxin (perfringolysin O), a cholesterol-binding cytolysin, with liposomal membranes: change in the aromatic side chains upon binding and insertion. *Biochemistry* **34,** 6513–6520.

Olson, R., Nariya, H., Yokota, K., Kamio, Y. and Gouaux, E. (1999). Crystal structure of Staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nature Struct. Biol*. **6,** 134–140.

Palmer, M. Vulicevic, I., Saweljew, P., Valeva, A., Kehoe, M. and Bhakdi. S. (1998). Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* **37,** 2378–2383.

Palmer, M. (2001). The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon* **39,** 1681–1689.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F. and Tsernoglou, D. (1994). Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* **367,** 292–295.

Pédelacq, J-D., Maveyraud, L., Prévost, G., Baba-Moussa, L., González, A., Courcelle, E., Shepard, W., Monteil, H., Samama, J-P. and Mourey, L. (1999). The structure of a *Staphylococcus aureus* leucocidin component (LukF-PV) reveals the fold of the water-soluble species of a family of transmembrane pore-forming toxins. *Structure* **7,** 277–287.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H. and Liddington, R.C. (1997). Crystal structure of the anthrax toxin protective antigen. *Nature* **385,** 833–838.

Pinkney, M., Beachey, E. and Kehoe, M. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun*. **57,** 2553–2558.

Polekhina, G., Giddings, K.S., Tweten, R.K. and Parker, M.W. (2004). Crystallization and preliminary x-ray analysis of the human specific toxin intermedilysin. *Acta Crystallogr. D* **60,** 347–349.

Polekhina, G., Giddings, K.S., Tweten, R.K. and Parker, M.W. (2005). Insights into the action of the superfamily of cholesterol-dependent cytolysins from studies of intermedilysin. *Proc. Natl. Acad. Sci. USA* **102,** 600–605.

Ramachandran, R., Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2002). Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. *Nature Struct. Biol*. **11,** 823–827.

Ramachandran, R., Tweten, R.K. and Johnson, A. E. (2004). Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit beta-strand alignment. *Nature Struct. Mol. Biol*. **11,** 697–705

Rood, J.I. (1998) Virulence genes of *Clostridium perfringens. Annu. Rev. Microbiol*. **52,** 333–360.

Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89,** 685–692.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4,** 406–425.

Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W. and Boulnois, G.J. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect. Immun*. **57,** 2547–2552.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Sekino-Suzuki, N., Nakamura, M., Mitsui, K.I. and Ohno-Iwashita, Y. (1996). Contribution of individual tryptophan residues to the structure and activity of θ-toxin (perfringolysin-O), a cholesterol-binding cytolysin. *Eur. J. Biochem*. **241,** 941–947.

Shepard, L.A., Heuck, A.P., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of a membrane-spanning domain of the thiol-activated, pore-forming toxin *Clostridium perfringens* perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. *Biochemistry* **37,** 14563–14574.

Shepard, L.A., Shatursky, O., Johnson, A.E. and Tweten, R.K. (2000). The mechanism of assembly and insertion of the membrane complex of the cholesterol-dependent cytolysin perfringolysin O: Formation of a large prepore complex. *Biochemistry* **39,** 10284–10293.

Song, L. Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of Staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Tweten, R.K. (1988) Nucleotide sequence of the gene for perfringolysin O (theta toxin) from *Clostridium perfringens* has significant homology with the genes for streptolysin O and pneumolysin. *Infect. Immun*. **56,** 3235–3240.

Tweten, R.K., Parker, M.W. and Johnson, A.E. (2001). The cholesterol-dependent cytolysins. *Curr. Top. Microbiol. Immunol*. **257,** 15–33.

Walker, J.A., Allen, R.L., Falmagne, P., Johnson, MK. and Boulnois, G.J. (1987). Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae. Infect. Immun*. **55,** 1184–1189.

CHAPTER

# 38

# Perfringolysin O and intermedilysin: mechanisms of pore formation by the cholesterol-dependent cytolysins

*Kara S. Giddings, Arthur E. Johnson, and Rodney K. Tweten*

## INTRODUCTION

Perfringolysin O (PFO) is a prototypic member of the large pore-forming toxin (PFT) family, the cholesterol-dependent cytolysins (CDCs). Members of the CDC family exhibit a high level of identity at the primary structure level, and therefore are predicted to share many similar features that define the CDC pore-forming mechanism. Of those features, two hallmark characteristics of this family include the formation of large, homo-oligomeric structures on the surface of the membrane (about 300 Å in diameter) and the absolute dependence of their cytolytic activity on the presence of membrane cholesterol (Heuck *et al*., 2001). The CDCs are produced as soluble monomers by various Gram-positive bacterial pathogens, but ultimately form a membrane-embedded, pore-forming oligomer. It is the transition from the soluble monomer to membrane pore and the associated structural changes in the monomer structure that have been one of the most intensively studied aspects of the CDCs in recent years. As will be discussed in this review, the study of the PFO mechanism has been key to understanding these structural transitions.

The ability of free cholesterol to abrogate toxin cytolytic activity has often been used to classify these toxins as CDCs. The inhibitory effect of adding free cholesterol to the CDCs was proposed to result from the occupation of a cholesterol-binding site on the toxin, thereby preventing it from binding to cholesterol-containing membranes (Alouf, 1999). Although this effect is well-known, the role of cholesterol in the mechanism of the CDCs has been difficult to elucidate. Recent studies on the novel CDC intermedilysin (ILY) suggest that the role of cholesterol in the CDC mechanism is more complex than originally proposed. ILY is unique among the CDCs in that it exhibits a restricted specificity for human erythrocytes (Nagamune *et al*., 1996). The ability of ILY to specifically target human cells suggested that it did not use cholesterol as its cellular receptor. Hence, ILY has proven to be a valuable tool for investigations into the role of cholesterol in the CDC mechanism, and its study has revealed some novel features that are incorporated into the CDC structure.

The material covered in this review will focus on aspects of the CDC cytolytic mechanism that have been elucidated in the last six years. These studies have led to the identification of several unique features that provide a more complete understanding of the fundamental changes in CDC structure that led to the assembly of the pore complex from the soluble CDC monomers. Furthermore, the discovery that the cytolytic activity of ILY was restricted to human cells has provided an important tool for the study of membrane recognition by the CDCs and the complex role of cholesterol in the CDC cytolytic mechanism.

## GENERAL FEATURES OF CDC STRUCTURE AND FUNCTIONAL MECHANISM

Specific details of CDC structure and function are provided in the following sections, but a brief overview will be provided here in order to orient the reader. The structure of the soluble monomer of PFO is shown in Figure 38.1 (see Chapter 37). PFO is largely comprised of β-sheet, and its structure can be divided into four domains. Domain 4 (D4) contains a highly conserved, tryptophan-rich undecapeptide. This peptide is highly conserved among the CDCs, but its role in pore formation remains elusive. It is clear that part of the undecapeptide inserts into the membrane (Nakamura *et al*., 1995; Heuck *et al*., 2003), though not to a significant depth (Heuck *et al*., 2003). In addition, three other short hydrophobic loops at the tip of D4 have been shown to insert into the membrane and help anchor PFO to the membrane (Ramachandran *et al*., 2002). It is also thought that the undecapeptide may interact directly with cholesterol, but a direct interaction between cholesterol and the undecapeptide has not been rigorously demonstrated.

After binding to the membrane, monomers diffuse laterally to initiate formation of the oligomer. Major structural rearrangements occur in D3 of PFO during this process. First, a loop comprised of a short β-strand and an α-helix must rotate away from one edge of the D3 core β-sheet to allow oligomerization of the monomers (Ramachandran *et al*., 2004). The monomers oligomerize into what has been termed a "prepore" complex (Shepard *et al*., 2000; Hotze *et al*., 2001). This complex resembles the pore complex, but it has not yet inserted its transmembrane β-barrel into the bilayer to form the pore. The transmembrane pore is formed when two α-helical bundles in D3 are converted to two extended amphipathic β-hairpins (Shepard *et al*., 1998; Shatursky *et al*., 1999) that insert into the membrane in a concerted fashion in the oligomer to form the pore (Hotze *et al*., 2002).

## CHOLESTEROL INVOLVEMENT IN THE CDC CYTOLYTIC MECHANISM

A fundamental issue in elucidating the mechanism of action for the CDC toxins is the role of membrane cholesterol. Numerous studies have previously implicated cholesterol as the membrane receptor for PFO and streptolysin O (SLO) and have equated the cholesterol-dependent inhibition of CDC activity to a blockage of CDC binding to receptor. It has been thought that occupation of the cholesterol-binding site on PFO (and other CDCs) prevents binding to membrane cholesterol. Ohno-Iwashita and co-workers have demonstrated that PFO can bind cholesterol directly and that PFO membrane binding is sensitive to the loss of membrane cholesterol (Ohno-Iwashita *et al*., 1986; Ohno-Iwashita *et al*., 1988; Iwamoto *et al*., 1990; Ohno-Iwashita *et al*., 1990; Ohno-Iwashita *et al*., 1991). However, the role of cholesterol in pore formation by the CDCs appears to be more complex than originally conceived.

Some evidence suggests that cholesterol may not control membrane recognition for all CDCs. One of the first observations to directly challenge the role of cholesterol as the CDC cellular receptor was a study by Jacobs *et al*. (Jacobs *et al*., 1998). They found that preincubation of LLO with exogenous cholesterol inhibited the cytolytic activity of the toxin, but did not appear to inhibit its binding to target membranes. They suggested instead that cholesterol affected a downstream event in pore formation by LLO, specifically oligomerization. The importance of cholesterol to CDC pore formation has also been examined for pneumolysin, a CDC produced by *Streptococcus pneumoniae*. Boulnois *et al*. (Boulnois *et al*., 1991) also suggested that cholesterol affected an event in the mechanism that occurred downstream of membrane binding. More recently, Billington *et al*. (Billington *et al*., 2002) showed that specific mutants of the CDC pyolysin (PLO), secreted by *Arcanobacterium pyogenes*, exhibited decreased cholesterol binding and cytolytic activity, but retained their ability to bind erythrocytes similarly to native toxin. But the most intriguing observation to cast doubt on the cholesterol-as-receptor dogma was made by Nagamune *et al*. (Nagamune *et al*., 1996). They found that the CDC intermedilysin (ILY) from *Streptococcus intermedius* formed pores only in human cells, an observation that was seemingly inconsistent with cholesterol serving as the CDC receptor.

Based on these previous studies, Giddings *et al*. (Giddings *et al*., 2003) examined the effect of membrane cholesterol depletion on the function of three different CDCs: PFO, ILY, and streptolysin O (SLO). They showed that the binding of SLO and ILY to the membranes was unaffected by the loss of approximately 90% of membrane cholesterol, while the cytolytic activity of both toxins on the same cholesterol-depleted erythrocytes was reduced greater than 99.9%. Under the same conditions, PFO binding was reduced approximately 10-fold in the absence of about 90% membrane cholesterol, yet PFO hemolytic activity was reduced by more than 11,000-fold (Giddings *et al*., 2003). Thus, the dependence of pore formation on cholesterol was

much greater than the dependence of protein binding to the membrane on cholesterol. It appears that the essential role of cholesterol in pore formation may be due primarily to its involvement in membrane insertion of the prepore complex and not membrane binding. In every case, the full activity of these toxins on the cholesterol-depleted membranes was restored by adding back cholesterol. Therefore, the loss of activity was due solely to the depletion of cholesterol from the membrane.

Giddings *et al*. (Giddings *et al*., 2003) determined that the loss of membrane cholesterol effectively stalled CDCs in a prepore complex. Therefore, the depletion of membrane cholesterol prevented the insertion of the transmembrane β-barrel. How cholesterol regulates the insertion of the CDC β-barrel remains unclear. It is possible that the effect of cholesterol on the bulk bilayer lipids (i.e., the formation of cholesterol-rich lipid rafts) provides conditions that facilitate the insertion of the β-barrel. An alternative but not mutually exclusive explanation is that a specific region of the CDC structure (presumably D4, see below) must interact with cholesterol to subsequently facilitate the conversion of the prepore to pore.

The work of Giddings *et al*. also explained how the cytolytic mechanism of the human cell–specific ILY could remain sensitive to cholesterol, but did not use it as a receptor. Therefore, their studies provided the impetus to explore the possibility that ILY used a nonsterol receptor to specifically recognize human cells. The ILY receptor is discussed below in detail.

## THE ROLE OF DOMAIN 4 IN CDC PORE FORMATION

### The interaction of domain 4 with the membrane

One of the most studied domains of PFO is D4. It is this domain that appears to interact with cholesterol, it contains the highly conserved undecapeptide sequence, and it is the first domain of the CDCs to interact with the membrane. Therefore, understanding its structure and function is key to understanding the subsequent structural changes that occur in the CDC molecule during pore formation.

In early studies, a proteolytically derived carboxy-terminal fragment of PFO (residues 305–500) containing all of D4 was shown to bind membrane independently of the intact PFO molecule (Iwamoto *et al*., 1990; Tweten *et al*., 1991). Based on crystal structure of PFO (Rossjohn *et al*., 1997) and location of D4 in the PFO molecule, it was postulated that D4 was not only responsible for the initial contact with the membrane, but that D4 penetrated the bilayer to generate the aqueous-lipid interface following pore formation (Rossjohn *et al*., 1997). Since that time, studies have unequivocally demonstrated that D3 of PFO harbors the peptide sequences that interact directly with the non-polar core of the bilayer during pore formation to create the β-barrel pore (Shepard *et al*., 1998; Shatursky *et al*., 1999) (see below).

Although D4 does not directly participate in the formation of the transmembrane β-barrel, it has been shown that the conserved undecapeptide and three other short hydrophobic loops at the tip of D4 insert into and anchor the monomer to the membrane (Heuck *et al*., 2000; Ramachandran *et al*., 2002). Collisional quenching studies showed that these loops did not penetrate deeply into the membrane and therefore were not involved in creating the pore, but instead were necessary to anchor the monomer to the bilayer and to orient it approximately perpendicularly to the membrane (Ramachandran *et al*., 2002). Furthermore, it appears that the surface of D4 that projects above the membrane is not in intimate contact with adjacent monomers in the CDC oligomeric pore complex and is, in fact, surrounded by the aqueous milieu (Ramachandran *et al*., 2002).

### Conformational coupling of domains 4 and 3

It is clear that the interaction of D4 with the membrane elicits subsequent structural changes that enable the membrane-bound monomers to oligomerize and form the pore structure. Heuck *et al*. first revealed that D3 and D4 are conformationally coupled in PFO, even though they are not in direct physical contact (Heuck *et al*., 2000). The interaction of PFO monomers with the membrane can be monitored by changes in the intrinsic fluorescence of the tryptophans within the undecapeptide region as they insert into the membrane (Nakamura *et al*., 1995). Kinetic analyses of the fluorescence changes associated with these tryptophans and with probes in D3 revealed that the interaction of D4 with the membrane preceded the insertion of the D3 transmembrane β-hairpins into the bilayer. Therefore, D4 interacts with the membrane surface before the transmembrane β-hairpins (Heuck *et al*., 2000). Interestingly, they also found that mutations in D3 that slowed the rate of insertion of the transmembrane β-hairpins also slowed the rate of association of D4 with the membrane. Since D4 interacted with the membrane before D3, the only explanation for this observation was that the two domains were conformationally coupled.

Although it is not unusual for two domains to be conformationally coupled, in this instance, the coupling

occurs even though the two domains are not in direct physical contact. Therefore, the coupling must require the involvement of D2, which is in contact with both domains 3 and 4, and possibly D1, which is in contact with D3 and D2. The results of these studies suggest that the undecapeptide and adjacent loops at the tip of D4 insert into the membrane, thereby anchoring the monomer firmly to the membrane. These changes in the D4 structure must trigger other changes in the structure of PFO outside of D4 that facilitate oligomerization into the prepore structure and modulate the insertion of the D3 transmembrane β-hairpins. Although the precise nature of the D3-D4 structural communication is not known, many aspects of the structural changes in the rest of the molecule that result in oligomerization and formation of the transmembrane β-barrel have been elucidated.

### Identification of a non-sterol membrane receptor for ILY

As described previously, it has been assumed for decades that cholesterol acts as the receptor for the CDCs. However, the recent studies of Giddings *et al*. (Giddings *et al*., 2003) have demonstrated that the major impact of cholesterol depletion from erythrocyte membranes on the CDC mechanism was on prepore to pore conversion, and not membrane binding. Hence, this finding showed that ILY activity remained sensitive to the presence of membrane cholesterol, but that cholesterol did not necessarily serve as a receptor for ILY. Nagamune *et al*. (Nagamune *et al*., 1996) showed that the activity of ILY was decreased on trypsin-treated human erythrocytes. Subsequently, Giddings *et al*. (Giddings *et al*., 2004) showed that under similar conditions of trypsinization, the binding of ILY was also decreased, further suggesting a non-sterol, proteinaceous receptor for this toxin. A detailed investigation by Giddings *et al*. (Giddings *et al*., 2003) into the interaction of ILY with human and non-human cells revealed that ILY utilizes human CD59 (huCD59) protein as its cellular receptor on the membrane surface.

CD59 is a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein that normally functions to inhibit the formation of the complement membrane attack complex (MAC) on host cells (Rollins and Sims, 1990) by binding to components of the MAC complex and thereby preventing the formation of the MAC pore (Chang *et al*., 1994; Lockert *et al*., 1995). CD59 specifically binds to complement proteins C8α and C9 of the MAC complex via a peptide region that includes residues 42–58 of the CD59 molecule (Zhao *et al*., 1998; Zhang *et al*., 1999). This peptide region exhibits the greatest level of heterogeneity between human, non-human primate, and animal CD59 molecules, and appears to be responsible for the species-selective inhibition of the complement MAC by CD59. The variability of this region between CD59 molecules of the various species also made it an ideal candidate to explain how ILY bound to huCD59, but not CD59 molecules from non-human species. Giddings *et al*. (Giddings *et al*., 2004) showed that the human cell specificity of ILY resulted from its recognition of this region of huCD59.

Giddings *et al*. (Giddings *et al*., 2004) also determined that D4 of ILY was sufficient for recognition and binding to huCD59. Using an ILY D4 glutathione-S-transferase (GST) fusion protein, they show that D4 alone could recognize and bind huCD59 similarly to native ILY. Furthermore, it appeared that the aberrant ILY undecapeptide was not involved in the cellular specificity of ILY (Nagamune *et al*., 2004).

These discoveries have altered how we view CDC membrane recognition and binding. It is now reasonable to propose that the first stage of the cytolytic mechanism of ILY, and perhaps other CDCs, involves the recognition of a cellular receptor other than membrane cholesterol. It also needs to be emphasized that although ILY does not use cholesterol as a receptor, it is clear from the above studies that its pore-forming mechanism is still highly dependent on the presence of membrane cholesterol. Therefore, the definition of these toxins as "cholesterol-dependent cytolysins" is the most appropriate nomenclature because it does not imply that these toxins must bind or use cholesterol as a receptor, but that their mechanism is still dependent on the presence of membrane cholesterol.

## FORMATION OF THE PREPORE

Several pore-forming toxins have been shown to first oligomerize on the cell surface into a prepore complex (Walker *et al*., 1995; Fang *et al*., 1997; Miller *et al*., 1999; Nguyen *et al*., 2002). The formation of a prepore complex by the CDCs is now well-established (Heuck *et al*., 2000; Shepard *et al*., 2000; Hotze *et al*., 2001; Hotze *et al*., 2002; Giddings *et al*., 2003; Heuck *et al*., 2003; Czajkowsky *et al*., 2004; Ramachandran *et al*., 2004). Generation of a prepore intermediate for PFO was first established by the observation that oligomer formation could proceed in the absence of pore formation at low temperatures (Shepard *et al*., 2000). Hotze *et al*. (Hotze *et al*., 2001) subsequently showed that a disulfide bond between D2 and the α-helical bundle that eventually

forms transmembrane β-hairpin 1 (TMH1) to D2 (Figure 38.1) effectively blocked pore formation, but not oligomerization of PFO. Reduction of this disulfide bond resulted in a rapid insertion of the transmembrane β-hairpins and formation of the pore complex. The size of the insertion-competent prepore complex is also large, and only large complexes form pores (Shepard *et al*., 2000; Hotze *et al*., 2002; Heuck *et al*., 2003) (see the next section for a complete discussion of the pore size). Hence, the CDCs form a large prepore complex prior to the insertion of the transmembrane β-barrel.

One of the features of all water-soluble, pore-forming toxin monomers secreted by bacteria is the necessity to undergo a structural change upon binding to the membrane in order to initiate oligomerization on the surface. Ramachandran *et al*. (Ramachandran *et al*., 2004) recently identified the structural rearrangement within the PFO monomer structure that is necessary to promote membrane oligomerization. They showed that a loop in D3 (Figure 38.1) blocks the association of monomers by preventing the hydrogen bonding of one β-strand at the edge of the core β-sheet of one D3 (β4) with a β-strand (β1) at the other edge of the D3 core β-sheet of a second monomer. This loop is comprised of a short β-strand (β5) and an α-helix. β5 forms hydrogen bonds with β4 of the D3 β-sheet and effectively blocks the interaction of β4 with β1 of a second monomer. However, when D4 binds to the membrane surface, a conformation change occurs more than 70Å above the surface that moves β5 away from β4 and thereby exposes β4 so that it can associate with β1 of another PFO molecule and initiate (or extend) oligomerization. Since monomers in solution (up to 20 mg/ml) do not associate or aggregate with each other, the short β5 loop acts as a dominant-negative regulator of oligomerization. Only after D4 binding to the membrane elicits a nearly coincident long-range allosteric conformational change far above the membrane can PFO monomers associate. These experiments (Ramachandran *et al*., 2004) also demonstrated that the edge-on interactions of the β1 and β4 strands of the D3 core β-sheet form a major interfacial region between the monomers in the membrane oligomer.

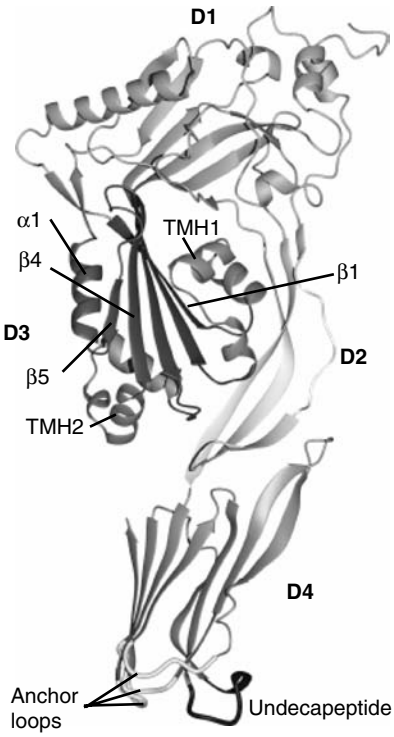



**FIGURE 38.1** The crystal structure of PFO. A ribbon representation of the crystal structure of the soluble monomer of PFO (Rossjohn, 1997) is shown. Shown are short hydrophobic loops of domain 4 (anchor loops) that have been determined to insert into the membrane surface (Ramachandran *et al*., 2002). The undecapeptide is labeled and shown in black. The two α-helical bundles (3 α-helices each) that ultimately form the twin transmembrane β-hairpins are labeled as TMH1 and TMH2 (**T**rans**M**embrane β-**H**airpin 1 and 2) (Shepard *et al*., 1998; Shatursky *et al*., 1999). The loop comprised of β-strand 5 (β5) and a short α-helix (α1) within domain 3 has been shown to block oligomerization of soluble PFO (Ramachandran *et al*., 2004). D1–D4: domains 1–4. The ribbon structure was created using the program MolMol (Koradi *et al*., 1996).

## PREPORE TO PORE CONVERSION

During prepore to pore transition each PFO monomer inserts two TMHs (TMH1 and TMH2, Figure 38.1) per monomer to create the large β-barrel pore (Shepard *et al*., 1998; Shatursky *et al*., 1999). Each β-hairpin is derived from an α-helical bundle in D3 that contains three short α-helices (Figure 38.1). This α-helical to β-strand transition in secondary structure is unique to the CDCs. Upon pore formation, these two α-helical bundles unravel to form the two extended β-hairpins that ultimately cross the membrane. This transition represents a major change in the monomeric structure of PFO as it makes the transition from membrane-bound monomer to a membrane-embedded oligomer. Although the structure of the soluble monomer and the membrane spanning β-hairpins of PFO were determined nearly six years ago, many of the molecular details of this transition have been revealed in only the past few years.

Hotze *et al*. (Hotze *et al*., 2002) first demonstrated that monomer-monomer interactions drive prepore to pore transition. They identified a mutation in PFO,

tyrosine 181 to alanine ($PFO^{Y181A}$) that trapped PFO in a prepore conformation. Yet, sufficient native PFO could force the TMHs of $PFO^{Y181A}$ to insert into the membrane in hybrid oligomers containing both wild-type and mutant PFO. In oligomers comprised of only $PFO^{Y181A}$, or a mixture of $PFO^{Y181A}$ and native PFO in which the concentration of native PFO was insufficient to overcome the barrier to insertion posed by the Y181A mutation, prepore to pore conversion did not occur. Thus, the conformational changes that occur in native monomers within the hybrid oligomer induce $PFO^{Y181A}$ to undergo the conformational change necessary for TMH insertion and pore formation. These data demonstrate that the monomers within the prepore complex act cooperatively to drive insertion of the transmembrane β-barrel into the bilayer.

Interestingly, the Y181A mutation led to the discovery of another important aspect of the prepore to pore conversion. Ramachandran *et al*. (Ramachandran *et al*., 2004) showed that the PFO β-barrel was successfully into the membrane only if tyrosine 181 formed a π-stacking interaction with phenylalanine 318 in β1 of the adjacent monomer. If either residue was substituted with a non-aromatic residue or its location altered by two residues (to position the amino acid side chain on the same side of the β-strand), the oligomer was trapped in the prepore state. A double mutant, in which the positions of Y181 and F318 were shifted to 179 and 320, respectively, maintained the relative alignment of the single aromatic residues in β1 and β4, and hence, the π-stacking interaction. This mutant was cytolytically active, further showing that the interaction of these two aromatic residues is important in aligning the adjacent TMHs and ensuring that all of the TMHs in the oligomer are in proper register prior to insertion. Having such a mechanism to prevent the misalignment of the TMHs is particularly important in a pore-forming complex that contains 35–50 monomers that must coordinately insert up to 100 TMHs into the membrane to form the β-barrel pore.

A central enigma in the mechanism of pore formation has been how the elongated TMHs were able to cross the bilayer from their location above the membrane surface. A previous study by cryoelectron microscopy of the membrane structure of another CDC, pneumolysin (PLY), concluded that the TMHs were approximately 40 Å too far from the membrane surface to successfully span the bilayer (Gilbert *et al*., 1999). However, it was clear from the studies of Shepard *et al*. (Shepard *et al*., 1998) and Shatursky *et al*. (Shatursky *et al*., 1999) that the TMHs of PFO were able to both traverse the bilayer and generate a β-barrel pore. However, Ramachandran *et al*. (Ramachandran *et al*., 2002) then showed that D4 was oriented perpendicular to the membrane. Since this arrangement of D4 more or less would position D3 far above the membrane unless the PFO conformation was altered from that of the soluble monomer, it was clear that additional conformational changes had to accompany pore formation.

Experimental demonstration of the major structural changes that occur during pore formation was reported by Czajkowsky *et al*. (Czajkowsky *et al*., 2004), who used atomic force microscopy (AFM) to examine PFO in the prepore and pore states. They showed that the height of the pore complex is 40 Å less than the height of the prepore complex. Hence, the prepore complex undergoes a 40 Å vertical collapse that brings the TMHs of D3 to the surface of the membrane and allows the TMHs to insert into and span the bilayer. They also showed that the lateral dimensions and topography of the prepore and pore oligomers were nearly identical, suggesting that there was no significant rotational "tipping over" of the monomers that would account for this collapse. Instead, the substantial reduction in the vertical height of the prepore complex is presumably achieved by a collapse of the D2 structure that allows D3 to move to the membrane surface. D2 is primarily comprised of two long β-strands whose structure is intimately associated with that of the D3 α-helical bundles that ultimately form TMH1. The stability of the D2 conformation is therefore very likely to be dependent on its interaction with the D3 α-helical bundles. If the D3 α-helices move away from D2 and undergo their transition to form TMH1 during the prepore to pore transition, the structural integrity of D2 would be compromised. This scenario is consistent with the absence of any significant difference in the topography of the prepore and pore structures in the plane of the membrane and would not require any change in the perpendicular orientation of D4 with the membrane.

The final stage of the pore-forming process is the production of a large membrane pore that is comprised of more than 35 monomers with a diameter of 250–300 Å. One still unknown question about pore formation is whether incomplete oligomeric rings can insert into the membrane and form a pore. Over the past 20 years, both circular oligomeric rings and incomplete rings (arcs) of CDCs have been observed on membranes using electron microscopy (Mitsui *et al*., 1979; Bhakdi *et al*., 1985). Although the size of the minimal pore-forming CDC complex remains ambiguous, it is clear that the prepore complex must reach an appreciable size before it inserts and forms a large pore (Heuck *et al*., 2000; Shepard *et al*., 2000; Hotze *et al*., 2001; Heuck *et al*., 2003). Liposome marker release assays per-

formed at limiting concentrations of PFO and SLO, in which an average of one pore per liposome was formed, showed that the rate of release was virtually identical for markers with substantially different hydrodynamic radii (≈ 10–140 Å) (Heuck *et al*., 2003). Since small molecules encapsulated in liposomes are released with the same kinetics as very large molecules, the holes formed by PFO and SLO do not appear to grow as a function of time. Instead, any hole formed is large enough to release both large and small molecules. Hence, PFO and SLO, and probably all CDCs, do not form small pores in membranes, and any small prepore complexes (i.e., small arcs) are apparently incapable of inserting into the membrane. It has been proposed that CDC molecules can insert individually into the bilayer rather than cooperatively as a complete circular prepore complex, thereby creating an intermediate state in which one side of the proposed hole is formed by exposed lipid, while the other side is formed by a CDC β-sheet in an arc (Palmer *et al*., 1998). While no direct evidence of arc insertion has yet been reported and the preponderance of evidence to date supports a concerted insertion mechanism, it remains unclear whether large, incomplete rings can make a transition from the prepore to pore, or if only complete rings are insertion competent.

## ROLE IN PATHOGENESIS

One of the most remarkable aspects of the CDCs is the size of the pore formed by these toxins in both mammalian cell membranes and also cholesterol-containing liposomes. It has always been somewhat puzzling as to why such a large pore is required if the primary use of these toxins is simple cell lysis. A much smaller pore, comprised of far fewer protein monomers, such as the *Staphylococcus aureus* α-hemolysin, would seem sufficient and would require much less energy to produce. The damaging effects of these toxins in terms of pore formation are easily recognized *in vitro*; however, the *in vivo* role of the CDC pore has been difficult to ascertain. The lysis of cells, particularly erythrocytes, has been a convenient model system for measuring the pore-forming activity of the CDCs. Yet, there has been no direct evidence to date that hemolysis or general cell lysis is a major role of the CDCs in bacterial pathogenesis.

The fact that the CDC pore is large enough to facilitate the passage of very large proteins suggests that the CDC pore can be used by bacterial pathogens in much more sophisticated ways. Madden *et al*. (Madden *et al*., 2001) showed that the SLO pore is used as a protein translocation channel by *Streptococcus pyogenes*. The SLO pore is utilized by *S. pyogenes* to permeabilize the membrane of keratinocytes and translocate a bacterial NAD-glycohydrolase enzyme (Spn) into the eukaryotic cytosol. In essence, the SLO pore acts as a Gram-positive bacterial analog of the Gram-negative bacterial type III secretion system. SLO differs structurally from other CDCs in that it contains about 80 additional amino acids at its amino terminus that are required for this translocation mechanism (Meehl and Caparon, 2004). Therefore, while the basic pore-forming mechanism is maintained, the SLO structure has evolved such that it can function as a protein translocation channel. This study is the first direct demonstration of a functional purpose for a CDC pore by a bacterial pathogen, and it also shows that the CDC structure is sufficiently adaptable that it can be employed to perform different tasks by changes in its structure.

Despite the progress made in understanding the individual steps that define the cytolytic mechanism of PFO, the importance of PFO to the development and progression of *C. perfringens* gas gangrene is less well understood. Both genetic and immunological studies have definitively established that α-toxin and PFO exhibit a synergistic effect in the development of gangrene. However, α-toxin is the primary virulence factor in this disease (Awad *et al*., 1995; Ellemor *et al*., 1999). Surprisingly, even though PFO is highly cytolytic, it does not appear to contribute significantly to the tissue necrosis (Awad *et al*., 1995). Apparently, PFO contributes to the leukostasis that results in the characteristic absence of polymorphonuclear leukocytes (PMNL) from the necrotic tissue in gas gangrene disease (Ellemor *et al*., 1999). More recently, it has been suggested that PFO could play a role in preventing macrophage-mediated clearing of *C. perfringens* (O'Brien and Melville, 2004). PFO appeared to be the primary agent responsible for killing macrophages, and the escape of *C. perfringens* from the macrophage phagosome was the consequence of PFO activity.

*S. intermedius* typically causes liver and brain abscesses, but the role of ILY in disease is not well understood. Due to the human cell specificity of ILY, it is unlikely that an animal model for *S. intermedius* disease will be developed in the near future. Indirectly, it has been shown that treatment of PMNL with ILY enhances the surface expression of the leukocyte integrin CD11b (Macey *et al*., 2001), which is also known as complement receptor 3. Defects in CD11b affect phagocytic cell adhesion and migration to the site of infection. Therefore, the excess of dysfunctional PMNL cells that is typically found in the abscesses of *S. intermedius* (Wanahita *et al*., 2002) might be the result of one or more effects of ILY. Also, ILY production in isolates

from deep-seated infections is 6–10 times higher than in isolates from the normal flora (Nagamune 2000). The ILY receptor human CD59 is up-regulated on the surface of activated PMNL (Gordon *et al*., 1994), and so during abscess formation, *S. intermedius* produces an excess of ILY that can then target a receptor-rich population of PMNL. Whether ILY directly lyses these cells or modifies their function in some way that is advantageous to the bacteria cell remains unknown.

## CONCLUSION

A number of major advances in understanding the CDC mechanism have been made in recent years by the study of the PFO and ILY. These studies have revealed many new paradigms for the CDC pore-forming mechanism and at the same time have changed our perspectives on long-held dogma. These and other studies have also shown that while the basic pore-forming mechanism is likely the same for the CDCs, their structures exhibit a plasticity that enables them to adapt and facilitate the pathogenic mechanism of the bacterial pathogen. In the future, the continued study of the CDC mechanism will reveal additional insights into the fundamental process of pore formation. It is also likely that more attention will now be focused on the structural plasticity of the CDCs that allows them to be customized for a specific purpose by the bacterial pathogen and how these pathogens use the toxins during disease development.

## ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (NIAID, AI037657).

## REFERENCES

Alouf, J.E. (1999). Introduction to the family of the structurally-related cholesterol-binding cytolysins ('sulfhydryl-activated toxins'). In: *Bacterial Toxins: A Comprehensive Sourcebook* (eds. J.E. Alouf and J.H. Freer), pp. 443–456. Academic Press, London.

Awad, M.M., Bryant, A.E., Stevens, D.L. and Rood, J.I. (1995). Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in Clostridium perfringens-mediated gas gangrene. *Mol. Microbiol*. **15,** 191–202.

Bhakdi, S., Tranum, J.J. and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin-O. *Infect. Immun*. **47,** 52–60.

Billington, S.J., Songer, J.G. and Jost, B.H. (2002). The variant undecapeptide sequence of the *Arcanobacterium pyogenes* hemolysin, pyolysin, is required for full cytolytic activity. *Microbiology* **148,** 3947–3954.

Boulnois, G.J., Paton, J.C., Mitchell, T.J. and Andrew, P.W. (1991). Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. *Mol. Microbiol*. **5,** 2611–2616.

Chang, C.P., Husler, T., Zhao, J., Wiedmer, T. and Sims, P.J. (1994). Identity of a peptide domain of human C9 that is bound by the cell-surface complement inhibitor, CD59. *J. Biol. Chem*. **269,** 26424–26430.

Cossart, P., Vincente, M.F., Mengaud, J., Baquero, F., Perez-Diaz, J.C. and Berche, P. (1989). Listeriolysin O is essential for the virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. *Infect. Immun*. **57,** 3629–3639.

Czajkowsky, D.M., Hotze, E.M., Shao, Z. and Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane β-hairpins to the membrane. *EMBO J*. **23,** 3206–3215.

Ellemor, D.M., Baird, R.N., Awad, M.M., Boyd, R.L., Rood, J.I. and Emmins, J.J. (1999). Use of genetically manipulated strains of *Clostridium perfringens* reveals that both alpha-toxin and theta-toxin are required for vascular leukostasis to occur in experimental gas gangrene. *Infect. Immun*. **67,** 4902–4907.

Fang, Y., Cheley, S., Bayley, H. and Yang, J. (1997). The heptameric prepore of a staphylococcal alpha-hemolysin mutant in lipid bilayers imaged by atomic force microscopy. *Biochemistry* **36,** 9518–9522.

Geoffroy, C., Gaillard, J.L., Alouf, J.E. and Berche, P. (1987). Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. *Infect. Immun*. **55,** 1641–1646.

Giddings, K.S., Johnson, A.E. and Tweten, R.K. (2003). Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *Proc. Natl. Acad. Sci. USA* **100,** 11315–11320.

Giddings, K.S., Zhao, J., Sims, P.J. and Tweten, R.K. (2004). Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. *Nat. Struct. Mol. Biol*. **12,** 1173–1178.

Gilbert, R.J.C., Jiménez, J.L., Chen, S., Tickle, I.J., Rossjohn, J., Parker, M., Andrew, P.W. and Saibil, H.R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*. *Cell* **97,** 647–655.

Gordon, D.L., Papazaharoudakis, H., Sadlon, T.A., Arellano, A. and Okada, N. (1994). Up-regulation of human neutrophil CD59, a regulator of the membrane attack complex of complement, following cell activation. *Immunol. Cell. Biol*. **72,** 222–229.

Heuck, A.P., Hotze, E., Tweten, R.K. and Johnson, A.E. (2000). Mechanism of membrane insertion of a multimeric β-barrel protein: Perfringolysin O creates a pore using ordered and coupled conformational changes. *Molec. Cell* **6,** 1233–1242.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2001). Beta-barrel, pore-forming toxins: intriguing dimorphic proteins. *Biochemistry* **40,** 9065–9073.

Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2003). Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J. Biol. Chem*. **278,** 31218–31225.

Hotze, E.M., Heuck, A.P., Czajkowsky, D.M., Shao, Z., Johnson, A.E. and Tweten, R.K. (2002). Monomer-monomer interactions drive the prepore to pore conversion of a beta barrel-forming, cholesterol-dependent cytolysin. *J. Biol. Chem*. **277,** 11597–11605.

Hotze, E.M., Wilson-Kubalek, E.M., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (2001). Arresting pore formation of a cholesterol-dependent cytolysin by disulfide trapping synchronizes the insertion of the transmembrane beta-sheet from a prepore intermediate. *J. Biol. Chem*. **276,** 8261–8268.

Iwamoto, M., Ohno-Iwashita, Y. and Ando, S. (1990). Effect of isolated C-terminal fragment of theta-toxin (perfringolysin-O) on toxin assembly and membrane lysis. *Eur. J. Biochem*. **194,** 25–31.

Jacobs, T., Darji, A., Frahm, N., Rohde, M., Wehland, J., Chakraborty, T. and Weiss, S. (1998). Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol. Microbiol*. **28,** 1081–1089.

Koradi, R., Billeter, M. and Wuthrich, K. (1996). MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph*. **14,** 51–55, 29–32.

Lockert, D.H., Kaufman, K.M., Chang, C.P., Husler, T., Sodetz, J.M. and Sims, P.J. (1995). Identity of the segment of human complement C8 recognized by complement regulatory protein CD59. *J. Biol. Chem*. **270,** 19723–19728.

Macey, M.G., Whiley, R.A., Miller, L. and Nagamune, H. (2001). Effect on polymorphonuclear cell function of a human-specific cytotoxin, intermedilysin, expressed by *Streptococcus intermedius. Infect. Immun*. **69,** 6102–6109.

Madden, J.C., Ruiz, N. and Caparon, M. (2001). Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152.

Meehl, M.A. and Caparon, M.G. (2004). Specificity of streptolysin O in cytolysin-mediated translocation. *Mol. Microbiol*. **52,** 1665–1676.

Miller, C.J., Elliot, J.L. and Collier, R.L. (1999). Anthrax protective antigen: prepore-to-pore conversion. *Biochemistry* **38,** 10432–10441.

Mitsui, K., Sekiya, T., Okamura, S., Nozawa, Y. and Hase, J. (1979). Ring formation of perfringolysin O as revealed by negative stain electron microscopy. *Biochim. Biophys. Acta* **558,** 307–313.

Nagamune, H., Ohkura, K., Sukeno, A., Cowan, G., Mitchell, T.J., Ito, W., Ohnishi, O., Hattori, K., Yamato, M., Hirota, K., Miyake, Y., Maeda, T. and Kourai, H. (2004). The human-specific action of intermedilysin, a homologue of streptolysin O, is dictated by domain 4 of the protein. *Microbiol. Immunol*. **48,** 677–692.

Nagamune, H., Ohnishi, C., Katsuura, A., Fushitani, K., Whiley, R.A., Tsuji, A. and Matsuda, Y. (1996). Intermedilysin, a novel cytotoxin specific for human cells secreted by *Streptococcus intermedius* UNS46 isolated from a human liver abscess. *Infect. Immun*. **64,** 3093–3100.

Nakamura, M., Sekino, N., Iwamoto, M. and Ohno-Iwashita, Y. (1995). Interaction of theta-toxin (perfringolysin O), a cholesterol-binding cytolysin, with liposomal membranes: change in the aromatic side chains upon binding and insertion. *Biochemistry* **34,** 6513–6520.

Nguyen, V.T., Higuchi, H. and Kamio, Y. (2002). Controlling pore assembly of staphylococcal gamma-hemolysin by low temperature and by disulphide bond formation in double-cysteine LukF mutants. *Mol. Microbiol*. **45,** 1485–1498.

O'Brien, D.K. and Melville, S.B. (2004). Effects of Clostridium perfringens alpha-toxin (PLC) and perfringolysin O (PFO) on cytotoxicity to macrophages, on escape from the phagosomes of macrophages, and on persistence of *C. perfringens* in host tissues. *Infect. Immun*. **72,** 5204–5215.

Ohno-Iwashita, Y., Iwamoto, M., Ando, S., Mitsui, K. and Iwashita, S. (1990). A modified θ-toxin produced by limited proteolysis and methylation: a probe for the functional study of membrane cholesterol. *Biochim. Biophys. Acta* **1023,** 441–448.

Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K., Ando, S. and Iwashita, S. (1991). A cytolysin, theta-toxin, preferentially binds to membrane cholesterol surrounded by phospholipids with 18-carbon hydrocarbon chains in cholesterol-rich region. *J. Biochem*. (Tokyo) **110,** 369–375.

Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K., Ando, S. and Nagai, Y. (1988). Protease nicked θ-toxin of *Clostridium perfringens*, a new membrane probe with no cytolytic effect, reveals two classes of cholesterol as toxin-binding sites on sheep erythrocytes. *Eur. J. Biochem*. **176,** 95–101.

Ohno-Iwashita, Y., Iwamoto, M., Mitsui, K., Kawasaki, H. and Ando, S. (1986). Cold-labile hemolysin produced by limited proteolysis of theta-toxin from *Clostridium perfringens. Biochemistry* **25,** 6048–6053.

Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J. and Bhakdi, S. (1998). Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. *EMBO J*. **17,** 1598–1605.

Portnoy, D., Jacks, P.S. and Hinrichs, D. (1988). The role of hemolysin for intracellular growth of *Listeria monocytogenes. J. Exp. Med*. **167,** 1459–1471.

Ramachandran, R., Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2002). Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. *Nat. Struct. Biol*. **9,** 823–827.

Ramachandran, R., Tweten, R.K. and Johnson, A.E. (2004). Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit β-strand alignment. *Nat. Struct. Mol. Biol*. **11,** 697–705.

Rollins, S.A. and Sims, P.J. (1990). The complement-inhibitory activity of CD59 resides in its capacity to block incorporation of C9 into membrane C5b-9. *J. Immunol*. **144,** 3478–3483.

Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89,** 685–692.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: A novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Shepard, L.A., Heuck, A.P., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of a membrane-spanning domain of the thiol-activated, pore-forming toxin *Clostridium perfringens* perfringolysin O: an α-helical to β-sheet transition identified by fluorescence spectroscopy. *Biochemistry* **37,** 14563–14574.

Shepard, L.A., Shatursky, O., Johnson, A.E. and Tweten, R.K. (2000). The mechanism of assembly and insertion of the membrane complex of the cholesterol-dependent cytolysin perfringolysin O: Formation of a large prepore complex. *Biochemistry* **39,** 10284–10293.

Tweten, R.K., Harris, R.W. and Sims, P.J. (1991). Isolation of a tryptic fragment from *Clostridium perfringens* θ-toxin that contains sites for membrane binding and self-aggregation. *J. Biol. Chem*. **266,** 12449–12454.

Walker, B., Braha, O., Cheley, S. and Bayley, H. (1995). An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch. *Chem. Biol*. **2,** 99–105.

Wanahita, A., Goldsmith, E.A., Musher, D.M., Clarridge, J.E., 3rd, Rubio, J., Krishnan, B. and Trial, J. (2002). Interaction between human polymorphonuclear leukocytes and *Streptococcus milleri* group bacteria. *J. Infect. Dis*. **185,** 85–90.

Zhang, H.F., Yu, J., Chen, S., Morgan, B.P., Abagyan, R. and Tomlinson, S. (1999). Identification of the individual residues that determine human CD59 species selective activity. *J. Biol. Chem*. **274,** 10969–10974.

Zhao, X.J., Zhao, J., Zhou, Q. and Sims, P.J. (1998). Identity of the residues responsible for the species-restricted complement inhibitory function of human CD59. *J. Biol. Chem*. **273,** 10665–10671.

CHAPTER

# 39

# Pneumolysin: structure, function, and role in disease

*Tim J. Mitchell*

## INTRODUCTION

Pneumolysin (PLY) is a hemolytic protein toxin produced by *Streptococcus pneumoniae* (the pneumococcus). The pneumococcus causes several important diseases of man including pneumonia, bacteraemia, meningitis, and otitis media. Infections with *S. pneumoniae* continue to have significant medical impact throughout the world (Ortqvist, 2001). The organism produces several potential virulence factors, including a polysaccharide capsule, a range of enzymes (hyaluronidase, neuraminidases, and superoxide dismutase), surface proteins (PspA and PsaA), and at least two hemolysins (Paton *et al*., 1993a; Canvin *et al*., 1997). There have also been three screens of the pneumococcal genome for virulence factors using signature-tagged mutagenesis (Polissi *et al*., 1998; Lau *et al*., 2001; Hava and Camilli, 2002), which identified hundreds of genes involved in its ability to survive in the murine host. Several virulence factors have been investigated with regard to their role in the pathogenesis of pneumococcal infection, and some can be considered as possible antigens for use in protective vaccination. There is obviously an interaction between virulence factors, and it has been demonstrated that there is an additive attenuation of the pneumococcus by the mutation of genes for PLY and other virulence factors (Berry and Paton, 2000). Many studies show that one of the key virulence factors produced by the pneumococcus is the pore-forming toxin PLY. This chapter will concentrate on PLY and will consider its mode of action, role in the disease process, and possible role in pneumococcal vaccines.

## HISTORICAL

Pneumococci were first reported to produce a hemolysin in 1905 (Libman, 1905). Since this report, numerous studies have been carried out using crude preparations of the toxin (Cohen *et al*., 1942; Cohen *et al*., 1940; Cole, 1914; Halbert *et al*., 1946; Neill, 1926; Neill, 1927), which demonstrated that the protein was toxic, susceptible to oxidation (a process that could be reversed by treatment with thiol-reducing agents), antigenic, and irreversibly inactivated by cholesterol. The sensitivity of the toxin to oxidation and reactivation with thiol-reducing agents led to the toxin being named "a thiol-activated toxin," a name that is now considered to be inappropriate. I will refer to this family as the "cholesterol-dependent cytolysins," or CDC. This family of proteins are produced by five genera of Gram-positive bacteria (Table 39.1). Recent advances in molecular biology and protein purification have now allowed large amounts of highly purified PLY to be produced and evaluated in a range of biological systems both *in vitro* and *in vivo*.

## STRUCTURE AND FUNCTION STUDIES OF PLY

### Mechanism of lytic action—the dogma

PLY is a lytic toxin able to lyse all cells that have cholesterol in their membranes. The mechanism of action of the members of this group of toxins are believed to be similar and has been reviewed (Morgan *et al*., 1996;

**TABLE 39.1** Cholesterol-dependent cytolysins and their primary amino acid sequence identity with PLY

| Organism | Toxin | % Identity with PLY amino acid sequence |
|---|---|---|
| **Streptococcus pneumoniae** | Pneumolysin | - |
| *Arcanbacterium pyogenes* | Pyolysin | 40 |
| *Bacillus alvei* | Alveolysin | 41 |
| *Bacillus anthracis* | Anthrolysin | 41 |
| *Bacillus cereus* | Cereolysin | 41 |
| *Clostridium perfringens* | Perfringolysin O | 45 |
| *Clostridium tetani* | Tetanolysin | 44 |
| *Listeria ivanovii* | Ivanolysin | 43 |
| *Listeria monocytogenes* | Listeriolysin O | 43 |
| *Listeria seelegeri* | Seeligerolysin | 43 |
| *Streptococcus intermedius* | Intermedilysin | 53 |
| *Streptococcus pyogenes* | Streptolysin O | 41 |
| *Streptococcus suis* | Suilysin | 50 |

Smyth and Duncan, 1978). Two of the major steps in the lytic process are membrane binding and oligomerization of the toxin to form pores. These two steps show differences in their temperature dependency. The binding of the toxin to cells is temperature independent, while oligomerization only occurs at higher temperatures. The lytic activity of the toxin can be inhibited by pre-incubation with cholesterol. This has led to the suggestion that membrane cholesterol is the receptor for this toxin. Once bound to the cell membrane, the toxin undergoes oligomerization to give high molecular weight pore-like structures composed of up to 50 monomeric units (Morgan *et al*., 1994). The exact mechanism of pore formation is still debated. Some studies suggest that these toxins can insert into the membrane and form pores by lateral diffusion (Palmer *et al*., 1998), while others suggest that the formation of a "pre-pore" is necessary prior to insertion into the membrane (Czajkowsky *et al*., 2004). Whatever the mechanism of insertion, these pores are believed to mediate the lysis of the cells by osmotic mechanisms. Inhibition of the lytic process by cholesterol is assumed to represent occupancy of the cholesterol binding site by free cholesterol preventing binding of the toxin to target cell membranes. However, studies with the related protein listeriolysin O suggest that inhibition of toxin action by cholesterol may not be due to a blockage of binding but to interference with the oligomerization process (Jacobs *et al*., 1998). More recently, similar experiments with PLY have shown that inhibition of the toxin with cholesterol does not prevent interaction with cells as measured by the PLY-induced production of gamma interferon (Baba *et al*., 2002). Also, it has been shown that for several members of this toxin family, depletion of membrane cholesterol traps the toxin in the prepore complex, suggesting that cholesterol is required for membrane insertion rather than binding (Giddings *et al*., 2003). The recent identification of CD59 as a protein receptor for intermidilysin (Giddings *et al*., 2004) also adds to the case that cholesterol may not serve as the primary receptor for all these toxins. Whether the contribution of cholesterol to the activity of PLY is solely as a receptor still remains to be determined.

## Analysis and modification of the primary amino acid sequence of PLY

PLY consists of a single 53kDa polypeptide chain and is produced by virtually all clinical isolates of the pneumococcus (Kanclerski and Mollby, 1987; Paton *et al*., 1983). PLY is the only member of this family of proteins that is not secreted from the cell but remains within the bacterial cytoplasm (Johnson, 1977). It has been shown, however, that some strains of the pneumococcus may be capable of active secretion of the toxin. Strain WU2 was shown to able to release PLY in the absence of autolytic activity (Balachandran *et al*., 2001).

The genes encoding PLY from serotype 1 and serotype 2 pneumococci have been cloned, sequenced, and expressed in *Escherichia coli* (Mitchell *et al*., 1989; Paton *et al*., 1986; Walker *et al*., 1987). Genes for PLY have also been cloned from several other serotypes, including from the genome sequence of a type 4 pneumococcus (Tettelin *et al*., 2001). Comparison of the derived amino acid sequences of these proteins shows a very high level of conservation with only a single amino acid change in the type 1 or type 4 sequence when compared to the type 2 sequence. Alignment of the amino acid sequence of PLY with the other members of the family shows there is a high degree of

similarity between the proteins (Table 39.1). Analysis of the primary amino acid sequence of PLY shows no major areas of hydrophobicity that could be involved in membrane insertion. The hydrophobic nature of PLY (Johnson *et al*., 1982) must therefore reflect the generation of hydrophobic areas during the folding of the primary sequence. In agreement with the cytoplasmic location of the toxin, the predicted amino acid sequence does not contain a signal sequence for secretion.

As PLY is thiol-activated, it was thought for many years that the activation process involved the reduction of an intramolecular disulphide bond (Smyth and Duncan, 1978). However, the primary amino acid sequence shows that PLY contains only a single cysteine residue. This residue lies at the C-terminal end of the molecule (amino acid position 428) in a region of 11 amino acids that is conserved among many of the family of toxins (Morgan *et al*., 1996). This conserved sequence (ECTGLAWEWWR) plays an important role in the activity of the toxin, as judged by studies using site-directed mutagenesis. Interestingly, the cysteine residue is not essential for activity and can be replaced with alanine with no effect on the lytic activity of the toxin *in vitro* (Saunders *et al*., 1989). However, the nature of the residue at this position is important, as substitution with glycine or serine results in a reduction in the activity of the toxin. Substitutions in other residues within the motif also affect the activity of the toxin, with changes in the tryptophan at position 433 having a dramatic effect. Attempts to define the nature of the defect in the mutants with reduced activity have been unsuccessful and have only determined that the effect is not due to a gross defect in the ability of the toxins to bind to cells or oligomerize (Saunders *et al*., 1989). It seems that the cysteine motif may play an important part in mediating a conformational change in the toxin when it interacts with membranes and allows the insertion of the toxin into the lipid bilayer.

Naturally occurring mutations in the amino acid sequence of PLY can also be informative. PLY derived from some serotype 8 pneumococci contains several alterations in amino acid sequence compared to the protein from other serotypes (Lock *et al*., 1996). The type 8 protein has substitutions at positions 172, 224, and 265 and has a two amino acid deletion (residues 270–271). Interestingly, these changes make the protein run with an apparent molecular weight on SDS-PAGE, which is greater than that from other more typical strains of the pneumococcus (Lock *et al*., 1996). Construction of chimeric molecules between the type 8 and the type 2 proteins demonstrated that it is the substitution at position 172 (threonine to isoleucine) that is responsible for the decreased activity of this version of the protein (Lock *et al*., 1996).

The mechanism by which PLY binds to cells is still unclear. The evidence that cholesterol is the receptor for the toxin is based on studies of the inhibition of toxin activity by prior incubation of the toxin with cholesterol in solution. The finding that the related toxin listeriolysin O is inactivated by cholesterol but is still able to bind to cells suggests that binding of these toxins to cells may involve a receptor other than cholesterol (Jacobs *et al*., 1998). Interaction with cholesterol may play a role in the assembly of the functional oligomer (Giddings *et al*., 2003). The region of the PLY molecule involved in cell binding has been inferred from studies using truncated versions of the protein (Owen *et al*., 1994). Removal of the five C-terminal amino acids of the toxin was sufficient to prevent cell lysis by the protein. A substitution of the proline residue at position 462 with serine causes a 90% reduction in the ability of the toxin to bind to red blood cells.

Studies with monoclonal antibodies have also been used to investigate the functional regions of PLY (de los Toyos *et al*., 1996). A panel of monoclonal antibodies was raised against PLY, all of which recognized linear epitopes of the protein. Three of these antibodies (termed PLY-4, PLY-5, and PLY-7) were capable of inhibiting the hemolytic activity of the toxin. One of the neutralizing antibodies (PLY-4) did not prevent binding of the toxin to cells, but did prevent the subsequent oligomerization of the toxin to form pores (as determined by electron microscopy), while the other two (PLY-5 and PLY-7) prevented cell binding by the toxin. Proteolytic treatment of PLY with proteinase K generates two fragments of 37kDa and 15kDa (Morgan *et al*., 1997). Purification and characterization of these fragments showed that the 15kDa fragment represented an N-terminal portion of the protein (K1), while the 37kDa fragment contained the remaining C-terminal part of the protein. The site of cleavage as determined by amino acid sequencing was between amino acids 142 and 143 (Morgan *et al*., 1997). Isolated K2 was still able to bind to liposomes and to form oligomer-like structures. Antibodies PLY-5 and PLY-7 both recognized the K2 portion of the protein, whereas PLY-4 recognized neither fragment. PLY-4 therefore recognizes at or near to the proteolytic cleavage site and prevents oligomerization. More recent studies have suggested that PLY-4 recognizes a conformational epitope, which includes the arginine residue at position 232 (Suarez-Alvarez *et al*., 2003). Further analysis of the binding site for PLY-5 using peptide mapping demonstrated that it recognizes the conserved undecapeptide region and prevents binding of the toxin to cells (Jacobs *et al*., 1999). PLY-7 recognizes residues 401–407 (Suarez-

Alvarez *et al*., 2003). The findings with monoclonal antibodies support the findings using truncated PLY that the C-terminus of the molecule is involved in cell binding. These findings are further supported by the observation that the purified C-terminal domain of PLY (amino acid residues 360–471) can still bind to cells (Baba *et al*., 2001).

The mechanism of pore formation by PLY is also still unclear. A recent paper by Czajkowsky *et al.* (2004) described the pre-pore to pore transition process. Site-directed mutations can be made in the conserved cysteine-containing region that have a dramatic effect on the lytic activity of the toxin, but no effect on the ability of the toxin to generate oligomers as observed by electron microscopy. In attempts to define the differences in the nature of the pore formed by wild-type PLY and a lytic-deficient mutant in which the tryptophan at position 433 has been replaced with phenylalanine (F433), the proteins have been studied in their effects on cells and on planar lipid bilayers (Korchev *et al*., 1998). The mutation at position 433 has no effect on the ability of the toxin to bind to cells or to form oligomeric structures as observed by electron microscopy. The mutation does affect the ability of the toxin to induce leakage of markers from Lettre cells and to induce conductance channels in planar lipid bilayers. PLY-induced leakage from Lettre cells is sensitive to inhibition by bivalent cations. The sensitivity to inhibition by bivalent cations was much reduced in the mutant form of the toxin. When inserted into planar lipid bilayers, PLY induces a range of conductance channels exhibiting small (less than 30pS), medium (30pS–1nS), and large (greater than 1nS) conductance steps. Small and medium channels are preferentially closed by bivalent cations. Wild-type toxin forms mainly small channels, whereas the mutant formed mostly large channels, which are insensitive to closure by divalent cations. Osmotic protection studies show that cells treated with wild-type toxin can be protected from lysis by polysaccharides with a molecular mass of more than 15kDa, whereas cells treated with the mutant form of the toxin were not protected with by polysaccharides with molecular mass over 40kDa. This also suggests that the functional pores are larger in the membranes treated with the F433 mutant. The cysteine motif therefore probably plays a role in the functionality of the pores generated by PLY. As no differences in the structure of the pores were observed by electron microscopy, the structural appearance of the pores does not reflect the functional state of the channels.

Purified PLY is also able to activate the classical complement pathway (Paton *et al*., 1984), and this effect has been linked to the ability of the toxin to bind to the Fc portion of human immunoglobulin (Mitchell *et al*., 1991). Analysis of the amino acid sequence of PLY showed it did not contain any homology to known IgG binding proteins, but did show some limited homology to the human acute phase protein C-reactive protein (CRP) (Mitchell *et al*., 1991). CRP is also able to activate the classical complement pathway, although this effect is mediated by a direct binding of complement component C1q (Volanakis and Kaplan, 1974). Mutagenesis of the homologous region in PLY revealed that residues 384 and 385 are involved in antibody binding and complement activation by the toxin (Mitchell *et al*., 1991). Subsequent analysis of a structural model of PLY based on the x-ray crystal structure of perfringolysin (Figure 39.1) (Rossjohn *et al*., 1998) showed there is no structural homology between the region of PLY and CRP. There is, however, a structural similarity between domain 4 of PLY and the Fc portion of antibody. As complement activation via the classical pathway is dependent on C1q binding to multimers of Fc, it is possible to suggest several mechanisms by which activation could occur. Aggregates of PLY could act to bind C1q in a similar manner to native Fc. Alternatively, the toxin may form mixed aggregates with immunoglobulins and activate complement. Certainly, the ability of the toxin to activate complement seems to be linked to its ability to interact with antibody (Mitchell *et al*., 1991). However, if PLY itself has structural similarities to immunoglobulin, it is possible that the original experiments measuring antibody binding are flawed. The molecular mechanism of complement activation by PLY requires further study.

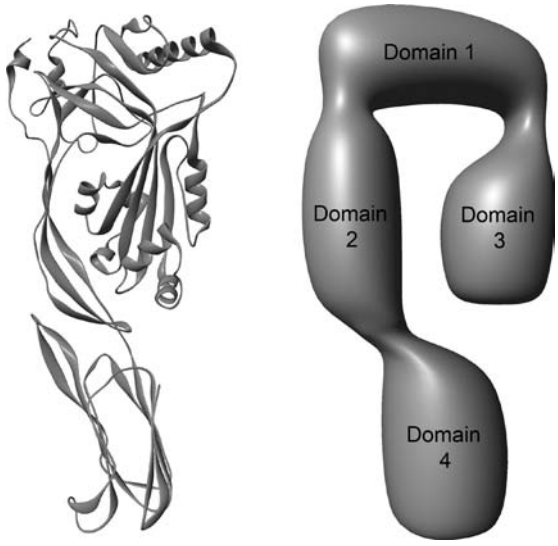



**FIGURE 39.1** Structural model of pneumolysin based on the crystal structure of perfringolysin (generated as described by Rossjohn *et al*. (Rossjohn *et al*., 1998)). Schematic illustrates the four domains of the protein. Figure generated by Graeme Cowan, University of Glasgow.

The studies described above allowed the generation of mutant versions of the PLY gene and proteins that have been instrumental in defining the roles of the various activities of the toxin during infection.

## BIOLOGICAL EFFECTS OF PLY

The biological effects of PLY are summarized in Table 39.2. PLY has been studied in a range of systems in attempts to define those properties that are important in the pathogenesis of infection. These systems include the study of the interaction of the purified protein with isolated cells, with organ cultures, and with whole animals. It is clear from the studies discussed below that the response of different cells and tissue to PLY can vary significantly. For example, the sensitivity of human monocytes and epithelial cells to PLY are different, and this difference may be affected by the activation status of the cells (Hirst *et al*., 2002).

The use of molecular genetic approaches has also allowed the role of the toxin to be addressed within the context of the whole organism via the construction of isogenic mutants that either do not express the protein or express a version that has an activity altered by modification of the toxin gene. These approaches have allowed detailed models to be built up of the role played by PLY in a range of experimental infections.

### Inflammatory properties of PLY

The effects of PLY on mouse macrophages appear to involve an interaction with Toll-like receptor 4 (TLR4) (Malley *et al*., 2003). Malley and co-workers (Malley *et al*., 2003) showed that the stimulation of macrophages to produce TNFα and IL-6 is dependent on the cytoplasmic TLR-adaptor molecule myeloid differentiation factor 88 (MyD88), and macrophages from mice with the targeted deletion of MyD88 did not respond to PLY. Purified PLY has a synergistic interaction with pneumococcal cell walls, which mediate inflammatory action via TLR2 (Yoshimura *et al*., 1999). Furthermore, when compared with wild-type macrophages, macrophages from mice carrying a spontaneous mutation in TLR4 were less responsive to both PLY alone and PLY in conjunction with pneumococcal cell wall. Interstingly, TLR4 mutant mice were more susceptible to lethal infection after intranasal colonization with pneumococci than were control mice. These findings show that interaction of PLY with the innate immune system via TLR-4 protects colonized animals from disease and suggests that inappropriate interaction with TLR4 could be responsible for some of the pathology seen in pneumococcal diseases such as pneumonia and meningitis.

Very low, sub-lytic concentrations of PLY can have dramatic biological effects on cells. The toxin has been shown to affect the production of pro-inflammatory mediators such as TNF-α, IL-1β, and IL-6

**TABLE 39.2** Selected biological activities of pneumolysin

| Activity | Reference |
|---|---|
| **Inflammation** | |
| Interaction with TLR4 | (Malley *et al*., 2003) |
| Production of cytokines from mononuclear cells | (Houldsworth *et al*., 1994) |
| Production of NO and activation of COX-2 | (Braun *et al*., 1999) |
| Activation of phospholipase A | (Rubins *et al*., 1994) |
| Proinflammatory effects on neutrophils | (Cockeran *et al*., 2001a; Cockeran *et al*., 2001b) |
| Enhancement of oxidative inactivation of alpha-1 proteinase inhibitor | (Cockeran *et al*., 2004) |
| Migration of T cells | (Kadioglu *et al*., 2004) |
| Microarray study of gene expression in mononuclear cells | (Rogers *et al*., 2003) |
| **Apoptosis** | |
| Macrophage apoptosis | (Dockrell *et al*., 2001; Marriott *et al*., 2004) |
| Neuronal apoptosis | (Braun *et al*., 2002; Stringaris *et al*., 2002) |
| **Effect on tissues** | |
| Pulmonary inflammation | (Feldman *et al*., 1991; Rijneveld *et al*., 2002) |
| Alveolar capillary permeability | (Rubins *et al*., 1993) |
| Cochlear damage | (Comis *et al*., 1993; Skinner *et al*., 2004) |
| Ependymal cells | (Mohammed *et al*., 1999; Hirst *et al*., 2004) |
| Eye | (Johnson and Allen, 1975) |

(Houldsworth *et al*., 1994) and stimulate nitric oxide (NO) production from macrophages, as well as increased transcription of the genes for COX-2 (Braun *et al*., 1999). COX-2 is the induced, rate-limiting enzyme involved in prostaglandin synthesis. The pathway by which PLY activates mouse macrophages is dependent on IFN-γ, as macrophages with a genetically inactivated IFN-γ signaling pathway are unable to produce NO in response to the toxin (Braun *et al*., 1999). PLY may have the ability to act as a general activator of macrophages, possibly through activation of NF-κB (Braun *et al*., 1999).

PLY can activate phospholipase A in pulmonary epithelial cells (Rubins *et al*., 1994). The activated phospholipase has a broad substrate specificity for cellular membrane phospholipids. Activation of phospholipase during an infection could contribute directly to lung damage by the release of free fatty acids and lysophophosphatides. Release of arachidonic acid by the activated phospholipase could promote chemotaxis and respiratory burst of neutrophils (Badwey *et al*., 1984; Curnutte *et al*., 1984). Arachidonic acid could also be metabolized through the eicosanoid pathway, leading to the production of leukotrienes and platelet activating precursor (Holtzman, 1991). Products of the eicosanoid pathway are also major chemotaxins for neutrophils (Cabellos *et al*., 1992).

The toxin interacts with neutrophils to cause increased production of superoxide, increased production of elastase, increased expression of $\beta_2$ integrins, and increased production of protaglandin $E_2$ and leukotriene $B_4$ (Cockeran *et al*., 2001a; Cockeran *et al*., 2001b). The toxin is therefore extremely pro-inflammatory. Cell recruitment may also be affected by PLY, as it has been shown to stimulate the production of IL-8 from human neutrophils (Cockeran *et al*., 2002). Thus, PLY has the potential to cause increased recruitment of inflammatory cells and increased production of pro-inflammatory mediators from these cells. The toxin has also been shown to potentiate the oxidative inactivation of alpha-1 proteinase inhibitor by activated neutrophils (Cockeran *et al*., 2004), which could lead to increased proteolytic activity in the lung and promote pneumococcal invasion. Activation of inflammatory pathways explains the ability of the toxin to induce pneumonitis similar to that induced by the whole organism (Feldman *et al*., 1991a). It seems somewhat of a paradox that the organism contributes to its own demise by producing this toxin and inducing inflammation. However, it may be that the inflammation induced is not effective in actually clearing the organism, as the toxin has also been shown to reduce killing of pneumococci by neutrophils *in vitro* (Paton and Ferrante, 1983). It might also be that inappropriate recruitment and activation of inflammatory cells by PLY contributes to pathology. However, recent studies using intratracheal administration of PLY to mice showed that the pneumotoxic effects observed were due to a direct effect of the toxin and not to resident or recruited phagocytic cells (Maus *et al*., 2004).

Recently, it has been suggested that pneumococci can induce the migration of CD4 T-cells *in vitro*. The migration induced by pneumococci grown *in vivo* was found to be greater than that by *in vitro* grown organisms. PLY is involved in the induction of the migration of CD4 T-cells, as PLN-A was unable to induce the effect (Kadioglu *et al*., 2004).

The effect of PLY on host gene expression has been examined using microarrays to compare the effect of wild-type pneumococci with PLY organisms on THP-1 mononulear cells (Rogers *et al*., 2003). Genes found to be up-regulated in response to WT but not PLY organisms include mannose-binding lectin 1, lysozyme, α-1 catenin, cadherin 17, caspases 4 and 6, macrophage inflammatory protein 1β (MIP-1β), interleukin 8 monocyte chemotactic protein 3 (MCP-3), IL-2 receptor β (IL-2Rβ), IL-15 receptor α (IL-15Rα), interferon receptor 2, and prosaglandin E synthase. Down-regulated genes include those for complement receptor 2/CD21, platelet-activating factor acetylhydrolase, and oxidized low-density lipoprotein receptor 1 (OLR1). These genes include representatives of functional genes involved in the anti-pathogen response, cell adhesion, cell cycle control, apoptosis, cell/cell signaling, lipid metabolism, protein degradation and folding, protein translocation, and signal transduction. PLY therefore has a dramatic effect on mononuclear cell function, some of which overlaps with known effects of the toxin (stimulation of IL-8 production, for example). The significance of most of these changes to the disease process remains to be determined.

## The role of PLY in apoptosis

*S.pneumoniae* has been reported to induce apoptosis in a number of cell types, including neutrophils, macrophages, and neuronal cells (Zysk *et al*., 2000; Dockrell *et al*., 2001; Braun *et al*., 2002; Stringaris *et al*., 2002). The contribution of PLY to the apoptotic process has been investigated. Zysk *et al*. (Zysk *et al*., 2000) report that PLY is responsible for mainly necrosis in human neutrophils and that apoptosis is induced by heat-killed bacteria. Dockrell *et al*. (Dockrell *et al*., 2001) used a PLY-negative mutant of the pneumococcus to show that apoptosis of macrophages infected with pneumococci was reduced in the absence of PLY. It has also been shown that macrophage apoptosis is mediated by nitric oxide and that this process is

induced by PLY to a greater extent by internalization of toxin-expressing bacteria than by exogenous toxin (Marriott *et al*., 2004). Apoptosis induced by PLY may be cell specific, as apotosis induced by the pneumococcus in lung epithelial cells is independent of the toxin (Schmeck *et al*., 2004).

As *S. pneumoniae* is a major cause of meningitis, a crucial interaction is between the organism and the blood-brain barrier (BBB). Using endothelial cells of cerebral origin as a model of the BBB, it has been shown that the majority of the damage inflicted on these cells is caused by PLY (Zysk *et al*., 2001). The damage induced by the toxin was dependent on protein synthesis, tyrosine phosphorylation, and caspase activity (Zysk *et al*., 2001).

The most detailed studies of the role of PLY in apoptosis have been done using neuronal tissues. Braun *et al*. (Braun *et al*., 2002) report that PLY can induce an apoptosis-inducing factor-dependent (AIF-dependent) form of apoptosis. Using a combination of bacterial mutants, it was shown that both PLY and hydrogen peroxide are responsible for the apoptosis of human microglial cells induced by *S. pneumoniae*. Ultrastructural analysis of human microglial cells showed that blocking the activity of PLY and hydrogen peroxide reduced the mitochondrial damage caused by the pneumococcus. The effects of the whole pneumococcus on microglial cells could be reproduced by addition of purified PLY, and the effect was dependent on the pore-forming activity of the toxin. PLY induces the increase of intracellular calcium levels and triggers the release of AIF from mitochondria in primary rat neurones. Pretreatment of microglial cells with the intra- and extra-cellular calcium chelator BAPTA-AM [1,2-bis (o-aminophenoxy) ethane N,N,N′,N′-tetraacetic acid tetra(acetomethoxyl)ester] blocked the release of AIF induced by PLY, suggesting that this event is mediated by increases in intracellular calcium levels. Finally, it was shown that PLY contributes to the neuronal damage in a rabbit model of pneumococcal meningitis.

An effect of PLY on calcium levels has also been demonstrated in neuroblastoma cells, where purified PLY was shown to induce apoptosis in a calcium-dependent manner (Stringaris *et al*., 2002). The changes in calcium levels were due to pore formation by the toxin, rather than opening of voltage-gated $Ca^{2+}$ channels. This study also showed that mitogen-activated protein kinase (MAPK) p38 was important in PLY-induced cell death.

## Interaction of PLY with animal tissue

Purified PLY has been evaluated for its effects on a range of tissues. When introduced into a ligated lobe of rat lung, the toxin induces histological features that are very similar to those observed in pneumonia (Feldman *et al*., 1991b). Intranasal inoculation of PLY into mice was associated with a dose-dependent influx of neutrophils, increased levels of interleukin-6, macrophage inflammatory protein-2, and KC into brochoalveolar lavage fluid (Rijneveld *et al*., 2002). When altered forms of the toxin are used, which have decreased lytic or complement-activating activity, the histological changes observed are much less than those induced by the wild-type toxin. This is an *in vivo* indication that the ability of PLY to lyse cells and to activate the complement pathway contributes to the pathogenesis of the inflammatory disease observed in the lung.

The effects of PLY on human respiratory mucosa have been investigated in an organ culture model. The toxin causes a slowing of the cilial beat of human nasal epithelium and at higher concentrations disrupts the integrity of the tissue (Feldman *et al*., 1990). This effect may be important in compromising the physical barrier of the mucocilary escalator during the development of an infection. PLY increases the alveolar permeability of perfused rat lungs and is toxic to type II rat alveolar epithelial cells (Rubins *et al*., 1993). The alveolar capillary barrier is essential in alveolar water and solute transport and may also provide a physical barrier to infection. Perturbation of this barrier by the toxin may play an important role in the development of pneumococcal pneumonia.

As well as causing pneumonia, the pneumococcus can also cause meningitis. A common complication of pneumococcal meningitis is hearing loss (Fortnum, 1992). The role of PLY in hearing loss has been investigated by direct perfusion of the toxin through the guinea pig cochlea. When the toxin was perfused through the scala tympani, widespread histological and electrophysiological damage was induced (Comis *et al*., 1993). Electrophysiological changes included reduced amplitude of both the compound action potential and the cochlear microphonic potential. These studies have recently been extended to show that the toxin has a preferential toxicity towards the inner hair cells of the cochlea (Skinner *et al*., 2004). Histological damage observed by scanning electron microscopy (Comis *et al*., 1993) includes damage to both inner and outer hair cells and supporting cells. Inner hair cells and outer hair cells of row three were most susceptible to the toxin. Damage to hair cells includes disruption and splaying of stereocilia, loss of stereocilia, and complete dissolution of hair bundles. The effects seen were dependent on the toxin concentration used, and at lower concentrations, the effects were reversible. The toxic effects of PLY on the guinea pig cochlea may be mediated, at least in part, by NO

(Amee *et al*., 1995). Pretreatment of the cochlea with $N^G$-methyl-L-arginine, a known inhibitor of NO synthesis, blocked the effect of the toxin. Also, pretreatment of the cochlea with MK-801, a N-methyl-D-aspartate (NMDA) receptor antagonist, also confers protection against PLY. This latter finding is consistent with the idea that excessive stimulation of NMDA receptors within the cochlea is responsible for the production of NO. The role of PLY in hearing loss has also been confirmed in an infection model of meningitis, and this will be discussed below. PLY may also perturb the membrane of the round window during pneumococcal otitis media and allow access of the toxin from the middle ear to the cochlea (Engel *et al*., 1995).

PLY has dramatic effects on the ciliated ependymal cells from the brains of rats. Brain cilia were more sensitive to the toxin than respiratory cilia and as little as 100ng/ml of toxin caused cilial stasis within 15 min (Mohammed *et al*., 1999). This concentration of toxin had no effect on respiratory cilia (Feldman *et al*., 1990). Ciliated cells line the ventricular surface of the brain and cerebral aqueducts and form a barrier between the CSF, which is infected during meningitis, and the brain tissue (Alfzelius, 1979). These cilia may protect the neuronal tissue from damage during infection by allowing continual movement of CSF and preventing migration of bacteria during meningitis. Perturbation of brain cilia by PLY could therefore compromise this defense mechanism. Use of both wild-type and PLY-deficient pneumococci in this system also caused ciliary slowing. However, treatment of wild-type but not PLY-deficient bacteria with penicillin increased the amount of ciliary slowing induced. The effect of antibiotic was blocked by anti-PLY antibodies. These studies show that antibiotic-induced lysis of pneumococci could have a detrimental effect in the brain and that antibodies to PLY may have therapeutic potential (Hirst *et al*., 2004).

The role of PLY in ocular infections has also been investigated. When the purified toxin is instilled into the eye of a rabbit, inflammatory response is generated (Johnson and Allen, 1975). The toxin induced similar pathology to that caused by natural infections with *S. pneumoniae*. If rabbits were made neutropenic prior to challenge with the toxin, the pathology induced was reduced, indicating that leukocytes may be a source of tissue-damaging enzymes such as collagenase (Harrison *et al*., 1993). The role of PLY in ocular infections has been confirmed in a rat model of endopthalmitis (Ng *et al*., 1997). In this study, purified PLY was injected intravitreally and induced many of the clinical and histopathologic features of pneumococcal endopthalmitis. The toxin therefore plays an important role in the inflammation and tissue damage that occurs in pneumococcal endopthalmitis. The role of PLY in ocular infection has also been investigated using infection models with genetically modified pneumococci. These studies will be considered below.

Purified PLY has been shown to affect a variety of cells and tissues. Major effects include compromise of host defence mechanisms and production of a potent inflammatory response. However, extrapolation from studies with a purified virulence factor to the role played by this virulence factor in the pathogenesis of an infection is not always valid. The generation of defined isogenic mutants of the pneumococcus that either do not produce the toxin or synthesize versions of the protein with altered biological activity allows the role played by the toxin in the pathogenesis of disease to be investigated in animal models of the diseases caused by *S. pneumoniae*.

## THE ROLE OF PLY IN PATHOGENESIS

### PLY-negative mutant

Although the existence of a toxin produced by the pneumococcus has been known for more than 90 years, it was not until the studies in James Paton's laboratory in the 1980s and early 1990s that it was shown that the toxin played an important role in the virulence of *S. pneumoniae*. The Paton group showed that immunization with a partially inactivated form of PLY could partially protect mice from challenge with virulent pneumococci (Paton *et al*., 1983). These studies formed the basis of a continuing evaluation of PLY toxoids as vaccine candidates (see below). The cloning and sequencing of the gene for PLY (Paton *et al*., 1986; Walker *et al*., 1987) allowed the construction of isogenic PLY-negative mutants of serotype 2 and serotype 3 pneumococci (Berry *et al*., 1989; Berry *et al*., 1992). In the case of serotype 2 pneumococci, the virulence of the organism was reduced by 100-fold when given intranasally to mice (Berry *et al*., 1989). When injected intravenously into mice, the PLY-negative mutant survived less well (Berry *et al*., 1989). The serotype 2 PLY-negative mutant was designated PLN-A, and this mutant has been key in studies of the role of PLY in the pathogenesis of disease in laboratories around the world.

The role of PLY in colonization of the nasopharynx appears to be strain- (or serotype-) dependent. PLY-sufficient pneumococci were able to colonize successfully and infect the nasophrynx, whereas PLY-deficient PLN-A was less able to colonize and tended to be cleared (Kadioglu *et al*., 2002). Differences in the contribution of PLY to colonization were observed when the

capsular serotype of the organism was changed (Kadioglu *et al*., 2002). This may explain other reports in the literature that suggest PLY plays no role in colonization (Rubins *et al*., 1998).

PLN-A has been used to define the role played by PLY in a mouse model of bronchopneumonia (Canvin *et al*., 1995). PLN-A induced much less inflammation when instilled into the mouse lung. Comparative histology of the inflammation induced by wild-type and pneumolysin-negative pneumococci is shown in Figure 39.2. This was an intriguing finding, as it had long been stated that the inflammation induced by pneumococci was a result of the release of inflammatory cell wall components (Tuomanen *et al*., 1987). Studies with PLN-A suggest that most of the inflammation is induced by the toxin. During pneumonia induced by the pneumococcus, there is a sequential influx of inflammatory cells into the lung (Kadioglu *et al*., 2000). In the absence of PLY, the inflammatory influx is delayed and less intense. The presence of PLY increases the rate of influx of neutrophils (Kadioglu *et al*., 2000). The toxin-negative mutant also showed a reduced ability to replicate in the lung. Invasion from the lung into the bloodstream was also delayed in the infection with PLN-A. In a recent study, the distribution of pneumococci in infected mice was monitored in real time using bioluminescent imaging (Orihuela *et al*., 2004). This study showed the contribution of various pneumococcal virulence factors to pathogenesis in whole animals to be tissue specific and that PLY is important after colonization in the growth of the pneumococcus in the lung and subsequent translocation to the bloodstream. Once in the bloodstream, PLY was required for high titer replication (Orihuela *et al*., 2004).

PLN-A has also been compared to wild-type pneumococci in a bacteremia model (Benton *et al*., 1995). These workers showed that PLY protected the pneumococcus from infection-induced host resistance. The wild-type parent organism exhibited exponential growth after intravenous injection until numbers reached as high as $10^{10}$ colony-forming units (CFU) per ml of blood when the animals died. When PLN-A was used, the initial growth rate was the same as wild-type until numbers of approximately $10^7$ CFU/ml were reached, when the increase in CFU/ml ceased, and the numbers of organisms in the blood remained constant for several days. If PLN-A was co-injected with wild-type organisms, it demonstrated wild-type growth characteristics. This observation suggests that PLY exerts its effect at a distance. In mice infected with PLN-A, there was evidence of an inflammatory response, including a rise in plasma levels of IL-6, a cessation of the net growth of PLN-A, and a control of the net growth of wild-type pneumococci, if given as a subsequent challenge. These data suggest that PLY allows the pneumococcus to cause acute sepsis rather than a chronic bacteremia. This effect seems to be limited to early times in the infection and once chronic bacteremia is established, PLY can no longer act as a virulence factor. Mice actively infected with wild-type pneumococcus produced more IL-6 per CFU in plasma than mice infected with comparable levels of PLN-A, suggesting that the increased resistance in PLN-A–infected mice is not mediated by IL-6 (Benton *et al*., 1995). Gamma interferon was only detected in mice

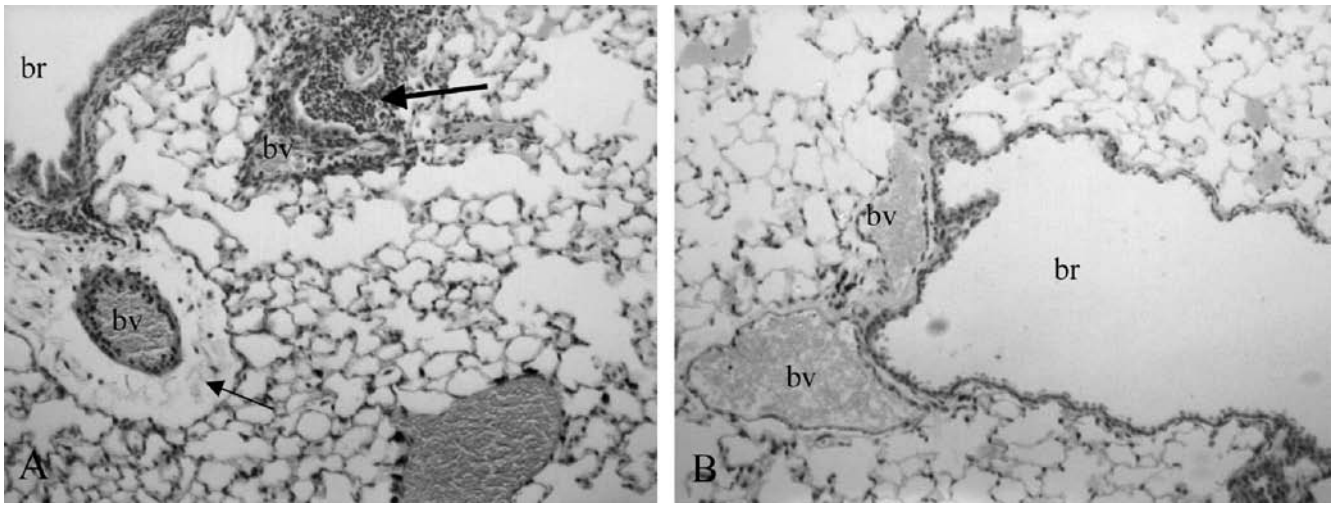



**FIGURE 39.2** Histology of lung 24 hours post-infection with (A) wild-type pneumococci and (B) pneumolysin-negative pneumococci. br: bronchioles, bv: blood vessels, large arrow: cell influx, small arrow: oedema. Figure generated by Alison Kerr, University of Glasgow.

infected with wild organisms that were near death from sepsis, suggesting this cytokine does not mediate the resistance. As PLY has been shown to mediate the production of tumor necrosis factor alpha (TNF-α) (Houldsworth *et al*., 1994), a study was undertaken to investigate the role of pro-inflammatory cytokines in the resistance to pneumococcal infection (Benton *et al*., 1998). These studies showed that the host resistance developed early during infection with PLN-A is dependent on TNF-α, but is independent of interleukin-1β or IL-6. The ability to cause acute sepsis in this model is therefore related to the ability to produce PLY. An inability to produce PLY is associated with chronic bacteremia and development of resistance to wild-type bacteria. This resistance is mediated by the production of TNF-α. The importance of the ability of PLY to stimulate TNF-α production is unclear in this model.

When evaluating the role played by PLY in infection, it is clear that the role played by the toxin differs according to the type of infection model used. In the pneumonia model described above, in which infection is established via the intra-nasal route of inoculation, the infection is typical of a bronchopneumonia. It is also possible to use a model of lobar pneumonia, in which the infection is established by intratracheal inoculation of mice (Rubins *et al*., 1995). When evaluated in this lobar pneumonia model, PLN-A was ten times less virulent than its wild-type parent. This model has been used to evaluate the possible role of PLY in pulmonary infection. Infection of animals with wild-type pneumococci caused an increase in leakage of serum albumin into the air spaces, indicating that the permeability of the alveolar capillary barrier had been compromised. PLY-negative pneumococci showed a decreased ability to grow in the air spaces of the lung and had much less effect on the permeability of the air/blood interface. Once in the lung tissue, the mutant organisms showed a decreased ability to grow within the lung tissue and to cause bacteremia. The virulence properties of PLN-A could be reverted to wild-type by the co-administration of a bolus of PLY. A mechanism by which PLY might affect the permeability of the alveolar capillary junction has been suggested in studies using human respiratory mucosa grown in organ culture (Rayner *et al*., 1995). The interaction of wild-type and PLN-A pneumococci with human respiratory mucosa in an organ culture with an air interface were studied for up to 48h. Beating of the cilia on the epithelium and adherence to and invasion of the epithelium by bacteria were monitored by scanning electron microscopy. Both wild-type and PLN-A caused a decrease in cilial beat frequency, although the onset of the inhibition was delayed in the case of PLN-A. This suggests that PLY is involved in causing cilial slowing, but is not the only factor involved. Histological damage was induced by both wild-type and PLN-A, but again the effect was delayed and less severe when the organisms did not produce PLY. Only the wild-type organism caused separation of tight junctions between epithelial cells, and at 48h post-infection wild-type pneumococci were adherent to the separated edges of otherwise healthy unciliated cells. This separation of tight junctions and adherence to exposed areas may account for the changes in permeability and tissue invasion in the lobar pneumonia model described above.

The interaction of a toxin with the host during pathogenesis of a bacterial infection is also influenced by the genetic status of that host. The interaction of PLY with mice genetically deficient in complement has been investigated (Rubins *et al*., 1995). Lobar pneumonia in complement-deficient mice showed several different features to that seen in complement-sufficient animals. Lack of a complete complement system was associated with increased numbers of bacteria in the lungs and an earlier and greater level of bacteremia. The clearance of PLN-A and especially wild-type pneumococci from lungs was reduced in complement-deficient animals. The total bacterial load increased in the complement-deficient lungs of PLN-A infected mice, whereas complement-sufficient mice were able to clear this organism. The effect of the removal of PLY expression can therefore be partially reversed by the use of complement-deficient mice, illustrating that the interaction of PLY with the complement system is important in these model systems.

The role of PLY in ocular infections with the pneumococcus has been investigated. When a deletion mutant lacking PLY was constructed and used in a rabbit model of ocular infection, it showed greatly reduced virulence (Johnson *et al*., 1990). A non-hemolytic strain of the pneumococcus produced by chemical mutagenesis (probably involving the generation of a point mutation in the PLY gene) was found to show the same virulence characteristics as the parent strain. This suggested that an activity of PLY other than its lytic function was important in the role of the toxin during pathogenesis of ocular infection. This was confirmed in studies using mutants of the toxin devoid of different activities (see below).

From the above, it is clear that PLY makes a contribution to the virulence of the pneumococcus in models of pulmonary, systemic, and ocular infections. Its role in some other diseases is less clear. Use of toxin-positive and toxin-negative isogenic pneumococci in the chinchilla model of otitis media showed that the amount of inflammation induced was similar (Sato *et al*., 1996). It should be pointed out that these studies were done

with a serotype 3 organism, whereas the studies described above with PLN-A are serotype 2. It may be that the contribution of PLY to virulence differs between serotypes. It is becoming increasingly clear that the contribution of various virulence factors to pathogenesis of pneumococcal diseases can differ between strains of the organism (Blue and Mitchell, 2003; McCluskey *et al*., 2004). In a study of the role of PLY in pathogenesis of pneumococcal meningitis, it was found that, although direct intracisternal injection of PLY into rabbits caused a rapid inflammatory response, there was no evidence of a contribution of the toxin to the inflammation caused by the whole organism as determined by infection with wild-type and PLN-A pneumococci (Friedland *et al*., 1995). Therefore, although PLY can stimulate the inflammatory cascade in the central nervous system, it is not necessary for the pathogenesis of meningeal inflammation. Wellmer *et al.* showed that a PLY-negative version of the pneumococcus was less virulent in a mouse model of meningitis (Wellmer *et al*., 2002). It has also been shown that the toxin plays no role in post-antibiotic enhancement of meningeal inflammation (Friedland *et al*., 1995). These findings with regard to inflammation were largely confirmed in the study of Winter and colleagues (Winter *et al*., 1997), who used a guinea pig model of pneumococcal meningitis. Use of wild-type and PLN-A pneumococci in this model showed there was no difference in the amount of inflammation induced. There was, however, less protein influx into the CSF of animals infected with PLN-A. This may be related to the decreased effect PLN-A is known to have on other cell barriers (discussed above for the alveolar/capillary barrier). Although no difference in inflammation was detected, PLN-A induced substantially less ultrastructural damage to the cochlea of infected animals, and there was less associated hearing loss. When meningitis was induced with PLN-A, there was almost no ultrastructural cochlear damage and significantly less hearing loss at all frequencies tested. The ultrastructural damage observed following infection with wild-type pneumococci was very similar to that induced by micro-perfusion of the cochlea with purified PLY (Comis *et al*., 1993). The ultrastructural changes induced by cochlear perfusion with PLY are those that were absent after meningitis due to PLN-A. Thus, in this experimental model, PLY appears not to contribute to inflammation, but the majority of cochlear damage and hearing loss is due to PLY. This also challenges the dogma that damage to the cochlea is due to bystander damage due to the inflammatory response. When considering the use of animal models of human disease, it is always useful to try to put things into a human context. The concentration of PLY in human CSF of human meningitis patients was found to be between 0.85 and 180ng/ml (Spreer *et al*., 2003). This is within the concentration range used in model systems to induce cytotoxity, inflammatory cytokine production, and alteration in cilial beat in both respiratory and ependymal mucosa (Spreer *et al*., 2003). Interestingly, this study also showed that the concentration of PLY in the CSF of rabbits with meningitis could be dramatically reduced by treatment with non-bacteriolytic antibiotics rather than ceftriaxone (Spreer *et al*., 2003).

The above describes some extensive studies on the role of PLY using simple isogenic knockout mutants. With the availability of molecular techniques, it has also proved possible to evaluate the contribution of individual activities or amino acids of the toxin to the virulence of the pneumococcus. These studies are described below.

## Administration of altered versions of purified PLY

As considered above, PLY is a multifunctional protein, and these various activities can be allocated to various parts of the protein. This process has been facilitated by the generation of a homology model of the three-dimensional structure of the toxin (Rossjohn *et al*., 1998). Structure function data for the protein can be used to define the roles played by the various activities of the toxin *in vivo*. Most studies have concerned the relative contribution of complement-activating activity and lytic activity to the pathogenesis of infection.

The first evidence that both complement activation and lytic activity contributed separately to the *in vivo* activity of the toxin was provided by Feldman and colleagues (Feldman *et al*., 1991b), who showed by direct instillation of purified PLY into ligated lobes of the rat lung that the molecule induced an inflammatory response similar to that seen in pneumonia. Use of mutant forms of PLY that lacked either lytic or complement activating activity demonstrated that both these activities contributed to the inflammatory process.

Studies by Rubins and colleagues (Rubins *et al*., 1995) using the rat lobar pneumonia model showed that full virulence could be restored to a PLY-negative mutant of the pneumococcus (PLN-A) by co-administration of a bolus of the wild-type toxin. Co-instillation of mutant forms of the toxin showed that the lytic activity of the toxin was important for allowing growth of bacteria in the lung and invasion into the lung tissue during the early times post-infection (up to 12h). Complement activation by the toxin only appeared to be important at later times.

### Isogenic mutants expressing altered versions of PLY

In order to understand how the various activities of PLY are involved in the virulence of the whole organism, a series of isogenic mutants of a type 2 pneumococcus were constructed that express versions of the toxin that carry various amino acid substitutions that affect the activity of the toxin (Berry *et al*., 1995). This panel of mutants has been used in several models of pneumococcal infection. The contribution of the various activities of the toxin to virulence varies according to the model used.

## ASSAY OF ISOGENIC STRAINS *IN VITRO*

Strains of pneumococci producing PLY, which differed in their ability to activate the complement pathway or cause cell lysis, have been used in assays of complement-mediated killing by human phagocytes to show that complement activation by the toxin is involved in modulating this process (Rubins *et al*., 1996b). Interaction with the complement system may well be important because it is involved in opsonophagocytosis and clearance of pneumococci from the lung (Winkelstein, 1981). Although the pneumococcal cell wall can activate the alternative complement pathway, the pneumococcal polysaccharide capsule is believed to prevent the access of phagocytes to these cell wall–bound opsonins (Winkelstein, 1981). It has been proposed that deposition of opsonins on the surface of the capsule is essential for effective opsonophagocytosis (Brown *et al*., 1983). The experiments with isogenic pneumococci suggest that PLY may be able to protect pneumococci from the classical complement pathway by consuming the components of the classical complement factors in solution. Evasion of the complement system in this way would allow increased bacterial survival, especially when the concentrations of complement factors are already low, such as in the lung.

## ASSAY OF ISOGENIC STRAINS *IN VIVO*: SYSTEMIC INFECTION

The virulence of the strains expressing toxins with altered activity have been compared in a model of intraperitoneal inoculation into mice (Berry *et al*., 1995). There was no significant difference in either median survival time or overall survival rate between mice challenged with wild-type or those with a deficiency in complement activation (strain H+/C–). Strains with reduced hemolytic activity (H2–/C+ and H3–/C+), which reduces the activity of the toxin produced to 0.02% and 0.0001%, respectively, had greater median survival times and overall survival rate than mice challenged with wild-type organisms. In the intraperitoneal model, the contribution of PLY to virulence is largely related to its cytotoxic properties rather than its ability to activate complement. It is interesting to note that very small amounts of hemolytic activity may be required for virulence, as strain H1–/C+ in which the lytic activity of the toxin is only 0.1% of wild-type had intermediate virulence.

The same isogenic strains have also been used in an intravenous challenge model (Benton *et al*., 1997). In this study, strains with decreased complement activation (H+/C–) or decreased lytic activity (H1–/C+, H3–/C+) had virulence characteristics similar to wild-type organisms. Only when mutations in the two activities were combined (H3–/C–) was there any effect on virulence. Moreover, the effect of these combined point mutations on virulence was small compared to that seen with the insertion duplication mutant PLN-A (PLY negative mutant). The PLN-A mutation increased survival time by 6 days, whereas the H3–/C– mutation only increased survival by 0.5 days. This suggests PLY has another activity other than lytic or complement activity that can contribute to virulence in this model. The existence of this third activity (in addition to complement activation and cytolysis) has been demonstrated by Berry *et al.* (Berry *et al*., 1999).

Use of a cirrhotic rat model of bacteremia revealed an important role for complement activation by PLY (Alcantara *et al*., 1999). In these studies, PLY-negative pneumococci and pneumococci expressing PLY without complement-activating activity were cleared from the bloodstream equally well and mortality observed was similar. However, PLY's complement-activating activity was proposed to enhance pneumococcal virulence in the hypocomplementemic, cirrhotic host (Alcantara *et al*., 1999). Under limiting concentrations of complement in the cirrhotic rat, the expression of PLY by pneumococci has a significant negative effect on serum complement levels and reduces serum opsonic activity (Alcantara *et al*., 2001).

## PNEUMONIA MODELS

In bronchopneumonia following intranasal inoculation of pneumococci, both lytic activity and complement activity are important, as reductions in these activities in the isogenic mutants reduced virulence (Alexander *et al*., 1998a). However, it was the ability to

activate complement that most affected the behavior of pneumococci in the lungs and associated bacteremia in the first 24h following infection. These findings were similar to those using a lobar pneumonia model (Rubins *et al*., 1996b), where it was confirmed using strains H3−/C+, H+/C−, and H3−/C− that either absence of lytic activity or complement-activating activity from PLY rendered mutants less virulent. This has been investigated further, and the cytolytic and complement-activating activities of the toxin have been shown to have different effects on cell recruitment (Jounblat *et al*., 2003). Complement activation by the toxin seemed to be involved in inducing T cell influx into the tissue, whereas cytolytic activity was responsible for neutrophil recruitment (Jounblat *et al*., 2003). In contrast to the bronchopneumonia model, these two mutations were not additive in their effect. The lack of additive effect in this system was proposed to show that the lytic activity of the toxin is involved in several steps during pathogenesis, whereas complement activity has a more limited role in reducing bacterial clearance in the lung. The lytic activity of the toxin correlated with acute lung injury and bacterial growth for up to six hours after inoculation. The complement-activating activity of the toxin correlated with bacterial growth and bacteremia at 24h post inoculation. Complement activation by PLY in lobar pneumonia has a singular role in pulmonary infection by promoting survival of pneumococci in the lung tissues and facilitating invasion in the blood (Rubins *et al*., 1996b).

## Summary of the role of PLY in pathogenesis

It is clear that PLY has different roles in different forms of pneumococcal disease. Thus, the toxin has no role in inflammation in meningitis (Friedland *et al*., 1995; Winter *et al*., 1997), but has a distinct role in meningitis-associated deafness (Winter *et al*., 1997) and bacteremia (Benton *et al*., 1995), as well as pneumonia. It also appears that where PLY has a role, the contributions of the hemolytic and complement-activating abilities differ. In bronchopneumonia, the hemolytic activity and complement activation by PLY each contribute to the virulence of pneumococci, and these activities have a distinct role in the disease (Alexander *et al*., 1998b). This is also true in lobar pneumonia (Rubins *et al*., 1996a) but with no additive effect on virulence when both activities were decreased, while in bronchopneumonia the survival time increased if both activities were reduced. After intraperitoneal infection, it was only changes that affected the lytic activity of the toxin that changed virulence (Berry *et al*., 1995), and the amount of hemolytic activity required for virulence was low. In the lung, hemolytic and complement-activating activities have distinct effects on the behavior of pneumococci, and the time at which each activity contributes is different. In bronchopneumonia, complement activation is important throughout the first 24h post-infection. Reduction in hemolytic activity only had an effect after 6h. In contrast, in lobar pneumonia, hemolytic activity was important in the first 6h, with complement activation only playing a role afterwards. It is probably significant that the inoculum entering the lungs is much smaller after intranasal inoculation (to establish bronchopneumonia), and the complement-activating ability of the toxin may be important in protecting this relatively small inoculum. When a large inoculum is used to establish lobar pneumonia, it is the ability to damage the alveolar capillary barrier to release nutrients that becomes important.

During bronchopneumonia, the level of bacteremia is closely related to the level of growth of bacteria in the lung. Mutations affecting growth in the lung (removal of complement-activating ability) therefore caused a corresponding decrease in the amount of bacteremia. Reduction of hemolytic activity had little effect on growth in the lung, and therefore a small effect on numbers of pneumococci in the blood. Studies using intravenous instillation of pneumococci into the blood suggest all mutants should grow at the same rate once into the blood (Benton *et al*., 1995; Berry *et al*., 1995; Benton *et al*., 1997). It therefore appears that continual seeding of bacteria from the lungs contributes to the level of bacteria in the blood rather than being due to a growth of an inoculum in the blood. An alternative explanation is that the wild-type and H−/C+ strain may be affected by passage through the lungs, i.e., entry to the blood via the lungs is different from entry to the blood via a syringe. Wild-type organisms injected into the blood reach levels of up to $10^{10}$ CFU/ml (Benton *et al*., 1995), whereas those entering the blood from the lung reach maximal levels of $10^8$ (Alexander *et al*., 1998a). This difference may be host mediated. Entry via the lungs may induce a host response, which controls subsequent bacteremia, whereas direct injection bypasses the induction of this response and allows greater growth of pneumococci. PLY may contribute to the down-regulation of this response when the organisms gain access to the lungs.

Pneumococci carrying three-point mutation in the PLY gene were not as avirulent as those making no toxin. This indicates that some further unidentified activity of PLY plays a role in infection (Benton *et al*., 1997). This could be related to the ability of strain H3−/C− to bind to the Fc portion of antibody (Mitchell, 1991; Alexander *et al*., 1998a), or could be related to the anticellular activity of sublytic concentrations of the toxin. It will be important to determine the nature of

the "unidentified" activity if PLY genetic toxoids are to be considered as vaccine candidates.

## REGULATION OF PLY EXPRESSION

Currently not much is known about how expression of the pneumolysin gene is regulated. However, with the advent of microarray technology, as well as techniques for the isolation of bacterial RNA from infected animals, this will soon change. Following intraperitoneal infection of BALC/c mice, the expression of PLY increased by threefold at 12h post infection and ten-fold at 24h post infection (Ogunniyi *et al*., 2002). The molecular mechanisms of this regulation are unclear, but the ClpP protease has been shown to play a role in posttranslational regulation of PLY *in vitro* (Kwon *et al*., 2004). Microarray analysis of a LuxS null mutant of strain D39 showed that LuxS plays a role in regulating the expression of PLY, although not in a cell density–dependent manner (Joyce *et al.,* 2004). These studies represent the first attempts to define the mechanisms of control of expression of this important virulence factor.

## USE OF PLY AS A VACCINE

The currently used pneumococcal vaccine for use in humans is based on a mixture of 23 capsular polysaccharides selected from the 90 known types. This vaccine has two major shortcomings. The first is that it is serotype specific, and the second is that protection against the types included may be very poor in high-risk groups who have a poorer antibody response to polysaccharide vaccines than healthy adults (Broome, 1981; Forrester *et al*., 1987). New conjugate vaccines have been developed containing the seven most common pediatric serotype polysaccharides conjugated to CRM197, and trials in Finland and the USA have demonstrated that these vaccines are efficacious in the prevention of invasive disease (Black *et al*., 2000; Eskola *et al*., 2001). However, these vaccines still protect only against seven serotypes, and they may drive selection of types not included in the vaccine. It has been proposed that a cross-serotype protective protein could overcome these problems. Protective pneumococcal antigens would also be attractive candidates for use as carriers for polysaccharides in conjugate vaccines. Conjugation of polysaccharides to proteins increases their immunogenicity and also converts them to antigens capable of generating immunological memory (Snippe *et al*., 1983). Studies with pneumococcal polysaccharides conjugated to a range of proteins have demonstrated enhanced immunogenicity of the polysaccharide (Andrew *et al*., 1994). The new generation of conjugate vaccines may be too expensive to be used in all parts of the world. A protein-based vaccine would therefore have attractions.

PLY is a good candidate as a vaccine antigen, as it is produced by virtually all clinical isolates of the pneumococcus (Kanclerski and Mollby, 1987), and its primary structure varies little (Mitchell *et al*., 1990). Mice can be protected from challenge with virulent pneumococci by passive immunization with either mouse monoclonal antibodies to PLY (Garcia-Suarez *et al*., 2004) or by purified human antibody to pneumolysin (Musher *et al*., 2001). Active vaccination with wild-type PLY is protective in mice (Paton *et al*., 1983), and combinations of PLY with other antigens, such as PspA, can increase protection further (Ogunniyi *et al*., 2000; Ogunniyi *et al*., 2001). Evidence for the production of PLY during invasive pneumococcal in humans is based on serological analysis. Bacteremic pneumococcal pneumonia is associated with a rise in anti-PLY titers in 50–85% of adults, especially in those with more severe pneumonia (Kalin *et al*., 1987; Kanclerski *et al*., 1988; Jalonen *et al*., 1989). Although native PLY is too toxic to be proposed for inclusion in a vaccine, genetically altered PLY with reduced toxicity could be used (Boulnois, 1992). The structure-function data and information on pathogenesis can be used for the rational design of candidate vaccine molecules. Immunization of mice with such a genetically engineered toxoid has been shown to confer protection against at least nine serotypes of the pneumococcus (Alexander *et al*., 1994). In this study, the amount of protection conferred was found to vary with the serotype of pneumococcus used and the route of challenge. No protection was afforded against the serotype 3 strain GBO5 when given intranasally. The absence of protection is not related solely to serotype, as some protection was afforded against another serotype 3 strain. The lack of protection against some pneumococcal isolates conferred by the PLY toxoid suggests that additional pneumococcal immunogens may be required in conjunction with the PLY toxoid. PLY could be used as a component of a multivalent protein vaccine, as an addition to a conventional conjugate vaccine, or as a carrier for pneumococcal polysaccharide. A conjugate of PLY toxoid and 19F polysaccharide has been produced and generates a protective immune response in infant mice (Lee *et al*., 1994a). Ply and toxoids of Ply have been used as carriers for polysaccharide in conjugates in several studies. The responses of animals to these vaccines are superior to those generated using other protein carriers in terms of antibody responses, neutralization of PLY, and protection of the animals for subsequent bacterial

challenge (Lee *et al*., 1994b; Kuo *et al*., 1995; Michon *et al*., 1998; Lee *et al*., 2001), indicating this is a potential route to improve vaccine efficacy. Alternatively, further protection may be achieved by alternative delivery routes of the PLY toxoid.

The experiments described above have considered only systemic immunity raised to the PLY toxoid. Since colonization of the nasopharyngeal mucosa by pneumococci is a prerequisite for the development of pneumococcal disease, it may be that mucosal immunity will give better protection from disease. Polysaccharides induce little secretory IgA at the mucosal surface and do not affect carriage rate of pneumococci (Douglas *et al*., 1986). The conjugate vaccines induce anti-capsular IgG and IgA antibodies in nasopharyngeal secretions (Nurkka *et al*., 2001), and immunization in infancy reduces carriage rates of the serotypes included in the vaccine. However, vaccination is accompanied by compensatory rises in the rates of nasopharyngeal colonization with serotypes not covered by the vaccine, and these may be associated with increased disease (at least for otitis media) caused by serotypes not included in the vaccine (Eskola, 2000). It may also be possible to generate a secretory IgA response to other pneumococcal antigens by mucosal immunization and thus influence colonization by virulent pneumococci. One mechanism for mucosal immunization is the use of live delivery vehicles. PLY has been expressed in such a delivery vehicle (Paton *et al*., 1993b). An attenuated *Salmonella* strain expressing PLY toxoid was able to elicit both IgG and IgA antibodies to the toxoid when given by the oral route (Paton *et al*., 1993b). New developments in DNA vaccine technology may also provide alternative routes and methods of vaccination with PLY.

## CONCLUSION

Many years' work have been done on PLY, such that much is now known about its activity. Detailed structure function analysis has revealed where within the molecule some of the biological properties of the toxin lie. A good homology model of the toxin now exists, although we still await a determination of the three-dimensional crystal structure of this toxin. The mechanism of pore formation by PLY is partially understood, but there are still many aspects to this intriguing process that remain to be clarified.

Availability of structure-function data and molecular biology technology in combination with traditional methods of studying pathogenesis has allowed a detailed analysis of the role of this toxin and its various activities in the pathogenesis of pneumococcal infection to be dissected. The role of the toxin is complex and varies according to which disease is examined. These studies highlight the difficulties in defining the role played by bacterial toxins in the disease process. Studies *in vitro* can only go some way to understanding the role of a toxin. Ultimate understanding can only be achieved by the study of *in vivo* systems.

## ACKNOWLEDGMENTS

I would like to thank all members of the Mitchell laboratory, both past and present, for their continued efforts to unravel the complexities of pneumolysin structure/function and biology. I would also like to thank the many collaborators around the world for continued productive interactions. Thanks to Alison Kerr and Graeme Cowan for their help with this chapter and the figures. Work in the Mitchell laboratory is supported by the Wellcome Trust, BBSRC, Royal Society, and the European Union.

## REFERENCES

Alcantara, R.B., Preheim, L.C. and Gentry, M.J. (1999). Role of pneumolysin's complement-activating activity during pneumococcal bacteremia in cirrhotic rats. *Infection and Immunity* **67,** 2862–2866.

Alcantara, R.B., Preheim, L.C. and Gentry-Nielsen, M.J. (2001). Pneumolysin-induced complement depletion during experimental pneumococcal bacteremia. *Infect. Immun*. **69,** 3569–3575.

Alexander, J.E., Berry, A.M., Paton, J.C., Rubins, J.B., Andrew, P.W. and Mitchell, T.J. (1998a). Amino acid changes affecting the activity of pneumolysin alter the behavior of pneumococci in pneumonia. *Microb. Pathog*. **24,** 167–174.

Alexander, J.E., Berry, A.M., Paton, J.C., Rubins, J.B., Andrew, P.W. and Mitchell, T.J. (1998b). The course of pneumococcal pneumonia is altered by amino acid changes affecting the activity of pneumolysin. *Microb. Pathogen*. **24,** 167–174.

Alexander, J.E., Lock, R.A., Peeters, C.C., Poolman, J.T., Andrew, P.W., Mitchell, T.J., Hansman, D. and Paton, J.C. (1994). Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae. Infect. Immun*. **62,** 5683–5688.

Alfzelius, B.A. (1979). The immotile-cilia syndrome and other ciliary diseases. *Int. Rev. Exp. Pathol*. **19,** 1–43.

Amee, F.Z., Comis, S.D. and Osbourne, M.P. (1995). $N^G$-methyl-L-arginine protects the guinea pig cochlea from the cytotoxic effects of pneumolysin. *Acta Otolaryngol* **115,** 386–391.

Andrew, P.W., Boulnois, G.J., Mitchell, T.J., Lee, C.-J., Paton, J.C., Poolman, J.T. and Peteers, C.C.A.M. (1994). Pneumococcal vaccines. *Zentral. Bakteriol*. **S24,** 453–466.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2001). Essential role of domain 4 of pneumolysin from *streptococcus pneumoniae* in cytolytic activity as determined by truncated proteins. *Biochemical and Biophysical Research Communications* **281,** 37–44.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2002). Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect. Immun*. **70,** 107–113.

Badwey, J.A., Curnutte, J.T., Robinson, J.M., Berde, C.B., Karnovsky, M.J. and Karnovsky, M.L. (1984). Effects of free fatty acids on release of superoxide and on change of shape by human neutrophils. Reversibility by albumin. *J. Biol. Chem*. **259,** 7870–7877.

Balachandran, P., Hollingshead, S.K., Paton, J.C. and Briles, D.E. (2001). The autolytic enzyme lytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *J. Bacteriol*. **183,** 3108–3116.

Benton, K., Everson, M. and Briles, D. (1995). A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. *Infect. Immun*. **63,** 448–455.

Benton, K.A., Paton, J.C. and Briles, D.E. (1997). The hemolytic and complement-activating properties of pneumolysin do not contribute individually to virulence in a pneumococcal bacteremia model. *Microb. Pathog*. **23,** 201–209.

Benton, K.A., VanCott, J.L. and Briles, D.E. (1998). Role of tumor necrosis factor alpha in the host response of mice to bacteremia caused by pneumolysin-deficient *Streptococcus pneumoniae. Infect. Immun*. **66,** 839–842.

Berry, A., Alexander, J., Mitchell, T., Andrew, P., Hansman, D. and Paton, J. (1995). Effect of defined point mutations in the pneumolysin gene on the virulence of Streptococcus pneumoniae. *Infect. Immun*. **63,** 1969–1974.

Berry, A.M., Ogunniyi, A.D., Miller, D.C. and Paton, J.C. (1999). Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect. Immun*. **67,** 981–985.

Berry, A.M. and Paton, J.C. (2000). Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect. Immun*. **68,** 133–140.

Berry, A.M., Paton, J.C. and Hansman, D. (1992). Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the viruelnce of *Streptococcus pneumoniae* type 3. *Microbial Pathogen*. **12,** 87–93.

Berry, A.M., Yother, J., Briles, D.E., Hansman, D. and Paton, J.C. (1989). Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae. Infect. Immun*. **57,** 2037–2042.

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J.R., Elvin, L., Ensor, K.M., Hacknell, J., Siber, G., Malinoski, F., Madore, D., Chang, I.H., Kohberger, R., Watson, W., Austrian, R. and Edwards, K. (2000). Efficacy, safety, and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. *Pediatr. Infect. Dis. J*. **19,** 187–195.

Blue, C.E. and Mitchell, T.J. (2003). Contribution of a response regulator to the virulence of *Streptococcus pneumoniae* is strain dependent. *Infect. Immun*. **71,** 4405–4413.

Boulnois, G.J. (1992). Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae. J. Gen. Microbiol*. **138,** 249–259.

Braun, J.S., Novak, R., Gao, G., Murray, P.J. and Shenep, J.L. (1999). Pneumolysin, a protein toxin of *Streptococcus pneumoniae*, induces nitric oxide production from macrophages. *Infect. Immun*. **67,** 3750–3756.

Braun, J.S., Sublett, J.E., Freyer, D., Mitchell, T.J., Cleveland, J.L., Tuomanen, E.I. and Weber, J.R. (2002). Pneumococcal pneumolysin and H2O2 mediate brain cell apoptosis during meningitis. *J. Clin. Invest*. **109,** 19–27.

Broome, C.V. (1981). Efficacy of pneumococcal polysaccahride vaccines. *Rev. Infect. Dis*. **3** (*supple*), S82–S88.

Brown, E.J., Joiner, K.A., Cole, R.M. and Berger, M. (1983). Localization of complement component C3 on *Streptococcus pneumoniae*: anti-capsular antibody causes complement deposition on the pneumococcal capsule. *Infect. Immun*. **39,** 403–409.

Cabellos, C., MacIntyre, D.E., Forrest, M., Burroughs, M., Prasad, S. and Tuomanen, E. (1992). Differing roles for platelet-activating factor during inflammation of the lung and sub-arachnoid space: the special case of *Streptococcus pneumoniae*. *J. Clin. Invest*. **90,** 612–618.

Canvin, J.R., Marvin, A.P., Sivakumaran, M., Paton, J.C., Boulnois, G.J., Andrew, P.W. and Mitchell, T.J. (1995). The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type-2 pneumococcus. *J. Infect. Dis*. **172,** 119–123.

Canvin, J.R., Paton, J.C., Boulnois, G.J., Andrew, P.W., and Mitchell, T.J. (1997). Streptococcus pneumoniae produces a second hemolysin that is distinct from pneumolysin. *Microb. Pathog*. **22,** 129–132.

Cockeran, R., Durandt, C., Feldman, C., Mitchell, T.J. and Anderson, R. (2002). Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils *in vitro. J. Infect. Dis*. **186,** 562–565.

Cockeran, R., Steel, H.C., Mitchell, T.J., Feldman, C. and Anderson, R. (2001a). Pneumolysin potentiates production of prostaglandin E2 and Leukotriene B4 by human neutrophils. *Infect. Immun*. **69,** 3494–3496.

Cockeran, R., Theron, A.J., Feldman, C., Mitchell, T.J. and Anderson, R. (2004). Pneumolysin potentiates oxidative inactivation of alpha-1-proteinase inhibitor by activated human neutrophils. *Respiratory Medicine* **98,** 865–871.

Cockeran, R., Theron, A.J., Steel, H.C., Matlola, N.M., Mitchell, T.J., Feldman, C. and Anderson, R. (2001b). Proinflammatory interactions of pneumolysin with human neutrophils. *J. Infect. Dis*. **183,** 604–611.

Cohen, B., Halbert, S.P. and Perkins, M.E. (1942). Pneumococcal hemolysin: the preparation of concentrates, and their action on red cells. *J. Bacteriol* **43,** 607–627.

Cohen, B., Perkins, M.E. and Putterman, S. (1940). The reaction between hemolysin and cholesterol. *J. Bacteriol*. **39,** 59–60.

Cole, R. (1914). Pneumococcus hemotoxin. *J. Exp. Med*. **20,** 346–362.

Comis, S.D., Osborne, M.P., Stephen, J., Tarlow, M.J., Hayward, T.L., Mitchell, T.J., Andrew, P.W. and Boulnois, G.J. (1993). Cytotoxic effects on hair cells of guinea pig cochlea produced by pneumolysin, the thiol activated toxin of *Streptococcus pneumoniae. Acta Otolaryngol* **113,** 152–159.

Curnutte, J.T., Badwey, J.M., Robinson, J.M., Karnovsky, M.J. and Karnovsky, M.L. (1984). Studies on the mechanism of superoxide release from human neutrophils stimulated with arachidonate. *J. Biol. Chem*. **259,** 11851–11857.

Czajkowsky, D.M., Hotze, E.M., Shao, Z. and Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane beta-hairpins to the membrane. *EMBO J*. **23,** 3206–3215.

de los Toyos, J., Mendez, F., Aparicio, J., Vazquez, F., Del Mar Garcia Suarez, M., Fleites, A., Hardisson, C., Morgan, P., Andrew, P. and Mitchell, T. (1996). Functional analysis of pneumolysin by use of monoclonal antibodies. *Infect. Immun*. **64,** 480–484.

Dockrell, D.H., Lee, M., Lynch, D.H. and Read, R.C. (2001). Immune-mediated phagocytosis and killing of *Streptococcus pneumoniae* are associated with direct and bystander macrophage apoptosis. *J. Infect. Dis*. **184,** 713–722.

Douglas, R.M., Hansman, D., Miles, H. and Paton, J.C. (1986). Pneumococcal carriage and type specific antibody. Failure of a 14-valent vaccine to reduce carriage in healthy children. *Am. J. Dis. Child*. **140,** 1183–1185.

Engel, F., Blatz, R., Kellner, J., Palmer, M., Weller, U. and Bhadki, S. (1995). Breakdown of the round window membrane permeability barrier evoked by streptolysin O: possible etiologic role in development of sensorineural hearing loss in acute otitis media. *Infect. Immun*. **63,** 1305–1310.

Eskola, J. (2000). Polysaccharide-based pneumococcal vaccines in the prevention of acute otitis media. *Vaccine* **19,** S78–S82.

Eskola, J., Kilpi, T., Palmu, A., Jokinen, J., Haapakoski, J., Herva, E., Takala, A., Kayhty, H., Karma, P., Kohberger, R., Siber, G. and Makela, P.H. (2001). Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N. Engl. J. Med*. **344,** 403–409.

Feldman, C., Mitchell, T.J., Andrew, P.W., Boulnois, G.J., Read, R.C., Todd, H.C., Cole, P.J. and Wilson, R. (1990). The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium *in vitro. Microb. Pathog*. **9,** 275–284.

Feldman, C., Munro, N.C., Jeffery, P.K., Mitchell, T.J., Andrew, P.W., Boulnois, G.J., Guerreiro, D., Rohde, J.A.L., Todd, H.C., Cole, P.J. and Wilson, R. (1991a). Pneumolysin induces the salient histologic features of pneumococcal infection in the rat lung *in vivo*. *American Journal of Respiratory Cell and Molecular Biology* **5,** 416–423.

Feldman, C., Munro, N.C., Jeffrey, D.K., Mitchell, T.J., Andrew, P.W., Boulnois, G.J., Gueirreiro, D., Rohde, J.A.L., Todd, H.C., Cole, P.J. and Wilson, R. (1991b). Pneumolysin induces the salient features of pneumococcal infection in the rat lung *in vivo. Am. J. Resp. Cell. Mol. Biol*. **5,** 416–423.

Forrester, H.L., Jahigen, D.W. and LaForce, F.M. (1987). Inefficacy of pneumococcal vaccine in high-risk population. *Am. J. Med*. **83,** 425–430.

Fortnum, H.M. (1992). Hearing impairment after bacterial meningitis: a review. *Arch. Dis. Child*. **67,** 1128–1133.

Friedland, I.R., Paris, M.M., Hickey, S., Shelton, S., Olsen, K., Paton, J.C. and McCracken, G.H. (1995). The limited role of pneumolysin in the pathogenesis of pneumococcal meningitis. *J. Infect. Dis*. **172,** 805–809.

Garcia-Suarez, M.d.M., Cima-Cabal, M.D., Florez, N., Garcia, P., Cernuda-Cernuda, R., Astudillo, A., Vazquez, F., De Los Toyos, J.R. and Mendez, F.J. (2004). Protection against pneumococcal pneumonia in mice by monoclonal antibodies to pneumolysin. *Infect. Immun*. **72,** 4534–4540.

Giddings, K.S., Johnson, A.E. and Tweten, R.K. (2003). Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *PNAS* **100,** 11315–11320.

Giddings, K.S., Zhao, J., Sims, P.J. and Tweten, R.K. (2004). Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. *Nat. Struct. Mol. Biol*. **11,** 1173–1178.

Halbert, S.P., Cohen, B. and Perkins, M.E. (1946). Toxic and immunological properties of pneumococcal hemolysin. *Bull. Johns Hopkins Hosp*. **78,** 340–359.

Harrison, J.C., Karcioglu, Z.A. and Johnson, M.K. (1993). Response of leukopenic rabbits to pneumococcal toxin. *Curr. Eye Res*. **2,** 705–710.

Hava, D. and Camilli, A. (2002). Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol. Microbiol*. **45,** 1389–1406.

Hirst, R.A., Mohammed, B.J., Mitchell, T.J., Andrew, P.W. and O'Callaghan, C. (2004). *Streptococcus pneumoniae*-induced inhibition of rat ependymal cilia is attenuated by antipneumolysin antibody. *Infect. Immun*. **72,** 6694–6698.

Hirst, R.A., Yesilkaya, H., Clitheroe, E., Rutman, A., Dufty, N., Mitchell, T.J., O'Callaghan, C. and Andrew, P.W. (2002). Sensitivities of human monocytes and epithelial cells to pneumolysin are different. *Infect. Immun*. **70,** 1017–1022.

Holtzman, M.J. (1991). Arachidonic acid metabolism. Implications of biological chemistry for lung function and disease. *Am. Rev. Resp. Dis*. **143,** 188–203.

Houldsworth, S., Andrew, P.W. and Mitchell, T.J. (1994). Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1β by human mononuclear phagocytes. *Infection and Immunity* **62,** 1501–1503.

Jacobs, T., Cima-Cabal, M.D., Darji, A., Mendez, F.J., Vazquez, F., Jacobs, A.A.C., Shimada, Y., Ohno-Iwashita, Y., Weiss, S. and de los Toyos, J.R. (1999). The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Letters* **459,** 463–466.

Jacobs, T., Darji, A., Frahm, N., Rohde, M., Wehland, J., Chakraborty, T. and Weiss, S. (1998). Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol. Microbiol*. **28,** 1081–1089.

Jalonen, E., Paton, J.C., Koskela, M., Kerttula, Y. and Leinonen, M. (1989). Measurement of antibody resonses to pneumolysin—a promising diagnostic method for the presumptive etiological diagnosis of pneumococcal pneumonia. *J. Infect*. **19,** 127–134.

Johnson, M.K. (1977). Cellular location of pneumolysin. *FEMS Micro. Lett*. **2,** 243–245.

Johnson, M.K. and Allen, J.H. (1975). The role of cytolysin in pneumococcal ocular infection. *Am. J. Opthalm*. **80,** 518–520.

Johnson, M.K., Hobden, J.A., Hagenah, M., OCallaghan, R.J., Hill, J.M. and Chen, S. (1990). The role of pneumolysin in ocular infections with *Streptococcus pneumoniae. Current Eye Research* **9,** 1107–1114.

Johnson, M.K., Knight, R.J. and Drew, G.K. (1982). The hydrophobic nature of thiol-activated cytolysins. *Bichem. J*. **207,** 557–560.

Jounblat, R., Kadioglu, A., Mitchell, T.J. and Andrew, P.W. (2003). Pneumococcal behavior and host responses during bronchopneumonia are affected differently by the cytolytic and complement-activating activities of pneumolysin. *Infect. Immun*. **71,** 1813–1819.

Joyce, E.A., Kawale, A., Censini, S., Kim, C.C., Couacci, A. and Falkow, S (2004). Lux S is Required for Persistant Pneumococcal Carriage and Expression of Virulence and Biosynthesis Genes. *Infect. Immun*. **72,** 2964–2975.

Kadioglu, A., Coward, W., Colston, M.J., Hewitt, C.R.A. and Andrew, P.W. (2004). CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal *Infection. Infect. Immun*. **72,** 2689–2697.

Kadioglu, A., Gingles, N.A., Grattan, K., Kerr, A., Mitchell, T.J. and Andrew, P.W. (2000). Host cellular immune response to pneumococcal lung infection in mice. *Infect. Immun*. **68,** 492–501.

Kadioglu, A., Taylor, S., Iannelli, F., Pozzi, G., Mitchell, T.J. and Andrew, P.W. (2002). Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect. Immun*. **70,** 2886–2890.

Kalin, M., Kanclerski, K., Granstrom, M. and Mollby, R. (1987). Diagnosis of pneumococcal pneumonia by enzyme-linked immunosorbent assay of antibodies to pneumococcal hemolysin (pneumolysin). *J. Clin. Micro*. **25,** 226–229.

Kanclerski, K., Blomquist, S., Granstrom, M. and Mollby, R. (1988). Serum antibodies to pneumolysin in patients with pneumonia. *J. Clin. Microbiol*. **26,** 96–100.

Kanclerski, K. and Mollby, R. (1987). Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). *J. Clin. Micro*. **25,** 222–225.

Korchev, Y.E., Bashford, C.L., Pederzolli, C., Pasternak, C.A., Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1998). A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers. *Biochem. J*. **329,** 571–577.

Kuo, J., Douglas, M., Ree, H. and Lindberg, A. (1995). Characterization of a recombinant pneumolysin and its use as a protein carrier for pneumococcal type 18C conjugate vaccines. *Infect. Immun*. **63,** 2706–2713.

Kwon, H.-Y., Ogunniyi, A.D., Choi, M.-H., Pyo, S.-N., Rhee, D.-K. and Paton, J.C. (2004). The ClpP protease of *streptococcus pneumoniae* modulates virulence gene expression and protects against fatal pneumococcal challenge. *Infect. Immun*. **72,** 5646–5653.

Lau, G.W., Haataja, S., Lonetto, M., Kensit, S.E., Marra, A., Bryant, A.P., McDevitt, D., Morrison, D.A. and Holden, D.W. (2001). A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. *Mol. Microbiol*. **40,** 555–571.

Lee, C.-J., Wang, T.R. and Frasch, C.E. (2001). Immunogenicity in mice of pneumococcal glycoconjugate vaccines using pneumococcal protein carriers. *Vaccine* **19,** 3216–3225.

Lee, C.J., Lock, R.A., Andrew, P.W., Mitchell, T.J., Hansman, D. and Paton, J.C. (1994a). Protection of infant mice from challenge with *Streptococcus pneumoniae* type 19F by immunization with a type 19F polysaccharide-pneumolysoid conjugate. *Vaccine* **12,** 875–878.

Lee, C.J., Lock, R.A., Andrew, P.W., Mitchell, T.J., Hansman, D. and Paton, J.C. (1994b). Protection of infant mice from challenge with *Streptococcus-pneumoniae* type 19f by immunization with a type 19f polysaccharide pneumolysoid conjugate. *Vaccine* **12,** 875–878.

Libman, E. (1905). A pneumococcus producing a peculiar form of hemolysis. *Proc. N.Y. Pathol. Soc*. **5,** 168.

Lock, R.A., Zhang, Q.Y., Berry, A.M. and Paton, J.C. (1996). Sequence variation in the *Streptococcus pneumoniae* pneumolysin gene affecting hemolytic activity and electrophoretc mobility of the toxin. *Microb. Pathogen*. **21,** 71–83.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R. and Golenbock, D.T. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *PNAS* **100,** 1966–1971.

Marriott, H.M., Ali, F., Read, R.C., Mitchell, T.J., Whyte, M.K. and Dockrell, D.H. (2004). Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection. *Faseb J*. **18,** 1126–1128.

Maus, U.A., Srivastava, M., Paton, J.C., Mack, M., Everhart, M.B., Blackwell, T.S., Christman, J.W., Schlondorff, D., Seeger, W. and Lohmeyer, J. (2004). Pneumolysin-induced lung injury is independent of leukocyte trafficking into the alveolar space. *J. Immunol*. **173,** 1307–1312.

McCluskey, J., Hinds, J., Husain, S., Witney, A. and Mitchell, T.J. (2004). A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in *Streptococcus pneumoniae. Mol. Microbiol*. **51,** 1661–1675.

Michon, F., Fusco, P.C., Minetti, C.A.S.A., Laude-Sharp, M., Uitz, C., Huang, C.-H., D'Ambra, A.J., Moore, S., Remeta, D.P., Heron, I. and Blake, M.S. (1998). Multivalent pneumococcal capsular polysaccharide conjugate vaccines employing genetically detoxified pneumolysin as a carrier protein. *Vaccine* **16,** 1732–1741.

Mitchell, T.J., Andrew, P.W., Saunders, F.K., Smith, A.N. and Boulnois, G.J. (1991). Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Mol. Microbiol*. **5,** 1883–1888.

Mitchell, T.J., Paton, J.C., Andrew, P.W. and Boulnois, G.J. (1990). Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2. *Nucleic Acids Research* **18,** 4010.

Mohammed, B.J., Mitchell, T.J., Andrew, P.W., Hirst, R.A. and Ocallaghan, C. (1999). The effect of the pneumococcal toxin, pneumolysin on brain ependymal cilia. *Microb. Pathog*. **27,** 303–309.

Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1996). Thiol-activated cytolysins. *Rev. Med. Micro*. **7,** 221–229.

Morgan, P.J., Harrison, G., Freestone, P.P.E., Crane, D., Rowe, A.J., Mitchell, T.J., Andrew, P.W. and Gilbert, R.J.C. (1997). Structural and functional characterization of two proteolytic fragments of the bacterial protein toxin, pneumolysin. *FEBS Lett*. **412,** 563–567.

Morgan, P.J., Hyman, S.C., Byron, O., Andrew, P.W., Mitchell, T.J. and Rowe, A.J. (1994). Modeling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. *J. Biol. Chem*. **269,** 25315–25320.

Musher, D.M., Phan, H.M. and Baughn, R.E. (2001). Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J. Infect. Dis*. **183,** 827–830.

Neill, J.M. (1926). Studies on the oxidation and reduction of immunological substrances. I. Pneumococcus hemotoxin. *J. Exp. Med*. **44,** 199–213.

Neill, J.M. (1927). Studies on the oxidation and reduction of immunological substrances. V. Production of anti-hemotoxin by immunization with oxidized pneumococcus hemotoxin. *J. Exp. Med*. **45,** 105–113.

Ng, E.W.M., Samily, N., Rubins, J.B., Cousins, F.V., Ruoff, K.L., Baker, A.S. and DAmico, D.J. (1997). Implication of pneumolysin as a virulence factor in *Streptococcus pneumoniae* endopthalmitis. *Retina* **17,** 521–529.

Nurkka, A., Ahman, H., Korkeila, M., Jantti, V., Kayhty, H. and Eskola, J. (2001). Serum and salivary anti-capsular antibodies in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. *Pediatr. Infect. Dis. J*. **20,** 25–33.

Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K. and Paton, J.C. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae. Infection and Immunity* **68,** 3028–3033.

Ogunniyi, A.D., Giammarinaro, P. and Paton, J.C. (2002). The genes encoding virulence-associated proteins and the capsule of *Streptococcus pneumoniae* are up-regulated and differentially expressed *in vivo. Microbiology* **148,** 2045–2053.

Ogunniyi, A.D., Woodrow, M.C., Poolman, J.T. and Paton, J.C. (2001). Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. *Infect. Immun*. **69,** 5997–6003.

Orihuela, C.J., Gao, G., Francis, K.P., Yu, J. and Tuomanen, E.I. (2004). Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J. Infect. Dis*. **190,** 1661–1669.

Ortqvist, A. (2001). Pneumococcal vaccination: current and future issues. *Eur. Respir. J*. **18,** 184–195.

Owen, R.H.G.O., Boulnois, G.J., Andrew, P.W. and Mitchell, T.J. (1994). A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae. FEMS Lett*. **121,** 217–222.

Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J. and Bhakdi, S. (1998). Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. *EMBO J*. **17,** 1598–1605.

Paton, J.C., Andrew, P.W., Boulnois, G.J. and Mitchell, T.J. (1993a). Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: The role of pneumococcal proteins. *Annu. Rev. Microbiol*. **47,** 89–115.

Paton, J.C., Berry, A.M., Lock, R.A., Hansman, D. and Manning, P.A. (1986). Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. *Infect. Immun*. **54,** 50–55.

Paton, J.C. and Ferrante, A. (1983). Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity and migration by pneumolysin. *Infect. Immun*. **41,** 1212–1216.

Paton, J.C., Lock, R.A. and Hansman, D.J. (1983). Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae. Infect. Immun*. **40,** 548–552.

Paton, J.C., Morona, J.K., Harrer, S., Hansman, D. and Morona, R. (1993b). Immunization of mice with Salmonella typhimurium C5 aroA expressing a genetically toxoided derivative of the pneumococcal toxin pneumolysin. *Microb. Pathog*. **14,** 95–102.

Paton, J.C., Rowan-Kelly, B. and Ferrante, A. (1984). Activation of human complement by the pneumococcal toxin pneumolysin. *Infect. Immun*. **43,** 1085–1087.

Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L. and Simon, D. (1998). Large-scale identification of virulence genes from *Streptococcus pneumoniae. Infect. Immun*. **66,** 5620–5629.

Rayner, C.F.J., Jackson, A.D., Rutman, A., Dewar, A., Mitchell, T.J., Andrew, P.W., Cole, P.J. and Wilson, R. (1995). Interaction of pneumolysin-sufficient and pneumolysin-deficient isogenic variants of *Streptococcus-pneumoniae* with human respiratory mucosa. *Infection and Immunity* **63,** 442–447.

Rijneveld, A.W., van den Dobbelsteen, G.P., Florquin, S., Standiford, T.J., Speelman, P., van Alphen, L. and van der Poll, T. (2002). Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice. *J. Infect. Dis*. **185,** 123–126.

Rogers, P.D., Thornton, J., Barker, K.S., McDaniel, D.O., Sacks, G.S., Swiatlo, E. and McDaniel, L.S. (2003). Pneumolysin-dependent and -independent gene expression identified by cDNA microarray analysis of THP-1 human mononuclear cells stimulated by *Streptococcus pneumoniae. Infect. Immun*. **71,** 2087–2094.

Rossjohn, J., Gilbert, R.J.C., Crane, D., Morgan, P.J., Mitchell, T.J., Rowe, A.J., Andrew, P.W., Paton, J.C., Tweten, R.K. and Parker, M.W. (1998). The molecular mechanism of pneumolysin, a virulence factor from *Streptococcus pneumonmiae. J. Mol. Biol*. **284,** 449–461.

Rubins, J.B., Charboneau, D., Fasching, C., Berry, A.M., Paton, J.C., Alexander, J.E. and Andrew, P.W. (1996a). Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *American Journal of Critical Care Medicine* **153,** 1339–1346.

Rubins, J.B., Charboneau, D., Fasching, C., Berry, A.M., Paton, J.C., Alexander, J.E., Andrew, P.W., Mitchell, T.J. and Janoff, E.N. (1996b). Distinct role for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *Am. J. Respir. and Critical Care Medicine* **153,** 1339–1346.

Rubins, J.B., Charboneau, D., Paton, J.C., Mitchell, T.J., Andrew, P.W. and Janoff, E.N. (1995). Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. *The Journal of Clinical Investigation*, 142–150.

Rubins, J.B., Duane, P.G., Clawson, D., Charboneau, D., Young, J. and Niewoehner, D.E. (1993). Toxicity of pneumolysin to pulmonary alveolar epithelial cells. *Infection and Immunity* **61,** 1352–1358.

Rubins, J.B., Mitchell, T.J., Andrew, P.W. and Niewoehner, D.E. (1994). Pneumolysin activates phospholipase A in pulmonary artery endothelial cells. *Infection and Immunity* **62,** 3829–3836.

Rubins, J.B., Paddock, A.H., Charboneau, D., Berry, A.M., Paton, J.C. and Janoff, E.N. (1998). Pneumolysin in pneumococcal adherence and colonization. *Microb. Pathog*. **25,** 337–342.

Sato, K., Quartey, M., Liebeler, C., Le, C. and Giebink, G. (1996). Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 Streptococcus pneumoniae strain in the chinchilla otitis media model. *Infect. Immun*. **64,** 1140–1145.

Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W. and Boulnois, G.J. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infection and Immunity* **57,** 2547–2552.

Schmeck, B., Gross, R., N'Guessan, P.D., Hocke, A.C., Hammerschmidt, S., Mitchell, T.J., Rosseau, S., Suttorp, N. and Hippenstiel, S. (2004). Streptococcus pneumoniae-induced caspase 6-dependent apoptosis in lung epithelium. *Infect. Immun*. **72,** 4940–4947.

Skinner, L.J., Beurg, M., Mitchell, T.J., Darrouzet, V., Aran, J.M. and Dulon, D. (2004). Intracochlear perfusion of pneumolysin, a pneumococcal protein, rapidly abolishes auditory potentials in the Guinea pig cochlea. *Acta Otolaryngol* **124,** 1000–1007.

Smyth, C.J. and Duncan, J.L. (1978). Thiol-activated (oxygen-labile) cytolysins. In: Bacterial Toxins and Cell Membranes, eds. J. Jeljaszewicz and T. Wadstrom, London: Academic Press, 129–183.

Snippe, H., Houte, A.-J.V., Dam, J.E.G.V., DeReuver, M.J., Jansze, M. and Willers, J.M.N. (1983). Immunogenic properties in mice of hexasaccharide from the capsular polysaccharide of *Streptococcus pneumoniae* type 3. *Infect. Immun*. **40,** 856–861.

Spreer, A., Kerstan, H., Bottcher, T., Gerber, J., Siemer, A., Zysk, G., Mitchell, T.J., Eiffert, H. and Nau, R. (2003). Reduced release of pneumolysin by *Streptococcus pneumoniae in vitro* and *in vivo* after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob. Agents Chemother*. **47,** 2649–2654.

Stringaris, A.K., Geisenhainer, J., Bergmann, F., Balshusemann, C., Lee, U., Zysk, G., Mitchell, T.J., Keller, B.U., Kuhnt, U. and Gerber, J. (2002). Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. *Neurobiology of Disease* **11,** 355–368.

Suarez-Alvarez, B., Garcia-Suarez, M.d.M., Mendez, F.J. and de los Toyos, J.R. (2003). Characterization of mouse monoclonal antibodies for pneumolysin: fine epitope mapping and V gene usage. *Immunology Letters* **88,** 227–239.

Tettelin, H., Nelson, K.E., Paulsen, I.T., Eisen, J.A., Read, T.D., Peterson, S., Heidelberg, J., DeBoy, R.T., Haft, D.H., Dodson, R.J., Durkin, A.S., Gwinn, M., Kolonay, J.F., Nelson, W.C., Peterson, J.D., Umayam, L.A., White, O., Salzberg, S.L., Lewis, M.R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A.M., Utterback, T.R., Hansen, C.L., McDonald, L.A., Feldblyum, T.V., Angiuoli, S., Dickinson, T., Hickey, E.K., Holt, I.E., Loftus, B.J., Yang, F., Smith, H.O., Venter, J.C., Dougherty, B.A., Morrison, D.A., Hollingshead, S.K. and Fraser, C.M. (2001). Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae. Science* **293,** 498–506.

Tuomanen, E., Rich, R. and Zak, O. (1987). Induction of pulmonary inflammation by components of the pneumococcal cell surface. *Am. Rev. Respir. Dis*. **135,** 868–874.

Volanakis, J.E. and Kaplan, M.H. (1974). Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requiremnet for human C1q. *J. Immunol* **113,** 9–17.

Walker, J.A., Allen, R.L., Falmagne, P., Johnson, M.K. and Boulnois, G.J. (1987). Molecular cloning, charcaterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae. Infect. Immun*. **55,** 1184–1189.

Wellmer, A., Zysk, G., Gerber, J., Kunst, T., von Mering, M., Bunkowski, S., Eiffert, H. and Nau, R. (2002). Decreased virulence of a pneumolysin-deficient strain of Streptococcus pneumoniae in murine meningitis. *Infect. Immun*. **70,** 6504–6508.

Winkelstein, J.A. (1981). The role of complement in the host's defense against *Streptococcus pneumoniae. Reviews of Infectious Disease* **3,** 289–298.

Winter, A., Comis, S., Osborne, M., Tarlow, M., Stephen, J., Andrew, P., Hill, J. and Mitchell, T. (1997). A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. *Infect. Immun*. **65,** 4411–4418.

Yoshimura, A., Lien, E., Ingalls, R.R., Tuomanen, E., Dziarski, R. and Golenbock, D. (1999). Cutting edge: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via toll-like receptor 2. *Journal of Immunology* **163,** 1–5.

Zysk, G., Bejo, L., Schneider-Wald, B.K., Nau, R. and Heinz, H.-P. (2000). Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae. Clin. Exp. Immunol*. **122,** 61–66.

Zysk, G., Schneider-Wald, B.K., Hwang, J.H., Bejo, L., Kim, K.S., Mitchell, T.J., Hakenbeck, R. and Heinz, H.-P. (2001). Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by *Streptococcus pneumoniae. Infect. Immun*. **69,** 845–852.

CHAPTER

# 40

# Listeriolysin

*José A. Vázquez-Boland, Radek Stachowiak, Lizeth Lacharme, and Mariela Scortti*

## INTRODUCTION

The cholesterol-dependent cytolysin (CDC) produced by the food-borne, facultative intracellular pathogen *Listeria monocytogenes* is known as listeriolysin O (LLO). Its coding gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria*. Subsequent characterization of the *hly* locus led to the discovery of *Listeria* pathogenicity island 1 (LIPI-1), a 9-kb chromosomal gene cassette encoding key functions necessary for the intracellular parasitic lifestyle of these bacteria. The elucidation between 1986 and 1989 of the crucial role that LLO plays in escape from the phagocytic vacuole made this toxin the first microbial product for which a function critical for the survival and replication of a parasite within host cells was identified. There is growing evidence that LLO is a multifaceted virulence factor with many other, sometimes subtle roles in the host-pathogen interaction. LLO displays all the common features of CDCs, but also unique properties making it a particularly interesting member of this toxin family. This chapter reviews the advances made in our understanding of the CDC with the best characterized role in pathogenesis. It also illustrates how this fundamental knowledge can be exploited to harness a nasty microbial product for the development of novel vaccines and therapeutic tools.

## CHARACTERIZATION

### Early studies

The production of a soluble hemolysin by *L. monocytogenes* was first reported by Harvey and Faber in 1941. During the 1960s and 1970s, various groups attempted the purification and characterization of this activity (Girard *et al*., 1963; Jenkins and Watson, 1971; Njoku-Obi *et al*., 1963; Siddique *et al*. 1974; Watson and Lavizzo, 1973). Jenkins *et al*., (1964) showed for the first time that the listerial hemolysin is functionally and antigenically related to streptolysin O (SLO), and Kingdon and Sword (1970) demonstrated two of its key characteristics: inhibition by cholesterol and pH optimum below 7. These authors also suggested that the *L. monocytogenes* hemolysin might be involved in the disruption of phagosomal membranes. Finally, Geoffroy *et al*. (1987) purified the toxin to homogeneity, demonstrated that it was a member of the CDC family, named it LLO, and determined that it was active at a narrow pH range, between 4.5 and 6.5 with optimum at 5.5. The 58-kDa CDC from *Listeria ivanovii*, ivanolysin O (ILO), was later purified and characterized (Vázquez-Boland *et al*., 1989a), and it was shown that the weakly hemolytic but non-pathogenic species *Listeria seeligeri* also produced an LLO-related CDC, albeit in small amounts (Geoffroy *et al*., 1989, Leimeister-Wächter *et al*., 1989).

### Molecular genetic studies

The *hly* gene encoding LLO was first identified and sequenced by Mengaud *et al*. (1988). Analysis of its deduced sequence and that of the cloned genes for ILO and the *L. seeligeri* hemolysin, seeligerilysin O (LSO) (Haas *et al*., 1992), revealed that the listerial hemolysins, although similar, are not identical (Figure 40.1). Their degree of similarity, 76–82% and 77–78% identity at the amino acid and nucleotide levels,

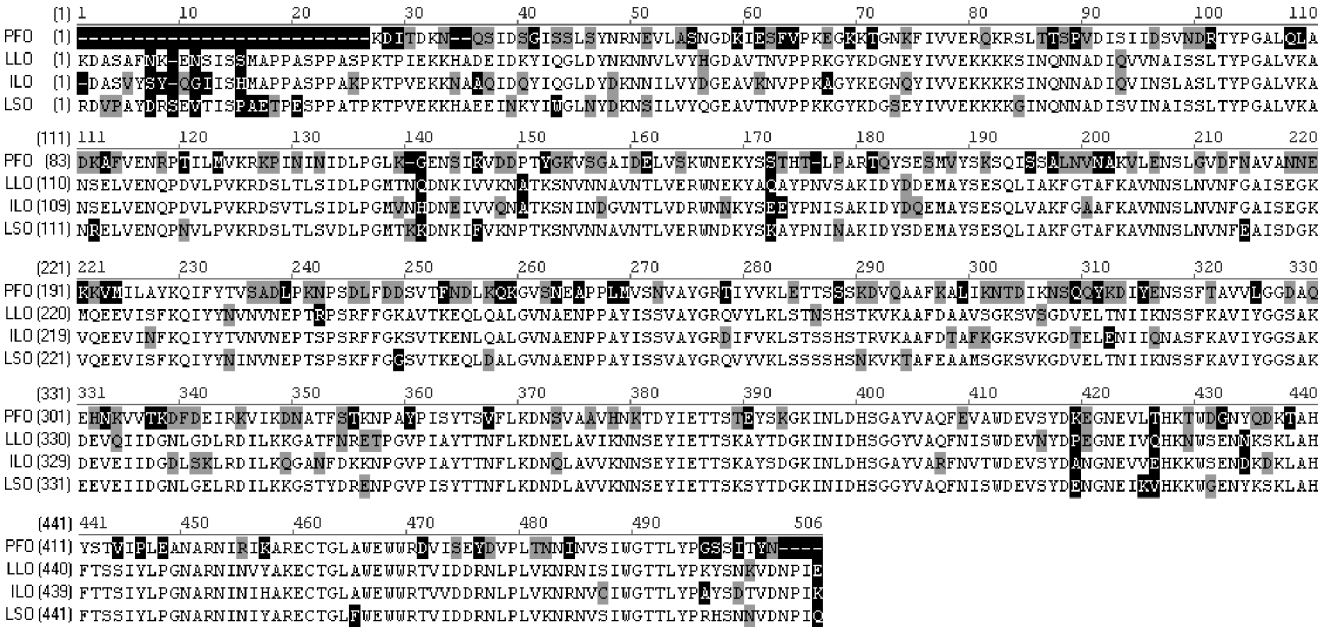


**FIGURE 40.1** Comparison of the amino acid sequeces of listerial CDCs (LLO, ILO, LSO) and PFO from *C. perfringens*. Differences in sequence are highlighted (light shade, conservative changes; dark shade, non-conservative changes). Multiple alignment of the mature proteins constructed with AlignX tool from VectorNTI Suite 9.0.0 package.

respectively, is comparable to that found between other orthologues shared by the three listerial species, consistent with the view that the central virulence gene cluster LIPI-1 bearing the hemolysin determinant (Figure 40.2) evolved vertically during speciation of the common *Listeria* ancestor (Vázquez-Boland *et al*., 2001a; Schmid *et al*., 2005).

## STRUCTURE-FUNCTION

### CDC structure and mechanism of pore formation

A crystal structure is available for the soluble monomer of perfringolysin O (PFO) (Rossjohn *et al*., 1997). Given the extensive overall similarity of CDCs in terms of amino-acid sequence and functional properties, it is reasonable to assume that the domain structure of PFO can be extrapolated to any member of this toxin family. Figure 40.3 depicts the theoretical three-dimensional structure of LLO modeled on the basis of the crystallographic data for PFO. LLO is predicted to consist of four discontinuous, predominantly β-sheet-folded domains spatially organized into two distinct protein regions: an N-terminal region formed by domains 1 to 3 and a C-terminal region formed by domain 4. Domains 1, 2, and 4 are aligned vertically, whereas domain 3 is located parallel to domain 2, forming an elongated mushroom-like molecule. The C-terminal domain 4 folds into a compact β-sandwich structure that is linked to the N-terminal region via residue Gly417 (Gly392 in PFO).

Membrane disruption by CDCs involves the transition from monomeric, water-soluble toxin molecules to non-covalently bound oligomeric, insoluble ring-shaped toxin structures formed by up to 50 monomers, which insert into the membrane, forming large pores about 20 to 30 nm in diameter (Bhakdi *et al*., 1985; Alouf and Geoffroy, 1991; Morgan *et al*., 1994; Sekiya *et al*., 1993) (Figure 40.4). A clear picture of the cytolytic mechanism of CDCs has begun to emerge from the integration of PFO structural data, site-specific mutagenesis analyses, and the information provided by biophysical and fluorescent probe–based studies performed with SLO, pneumolysin (PLY), and particularly PFO (Rossjohn *et al*., 1997; Nakamura *et al*., 1998; Palmer *et al*., 1998a, 1998b; Shepard *et al*., 1998, 2000; Gilbert *et al*., 1999; Shatursky *et al*., 1999; Heuck *et al*., 2000, 2003; Ramachandran *et al*., 2002, 2004) (see relevant chapters in this book for more information). These studies suggest that domain 4 mediates membrane binding and domains 1–3 are involved in toxin oligomerization and membrane disruption. This is consistent with experimental data obtained with LLO showing that neutralizing antibodies against the native toxin that map to domain 4 inhibit binding to cellular membranes (De los Toyos *et al*., 1996), whereas neutral-

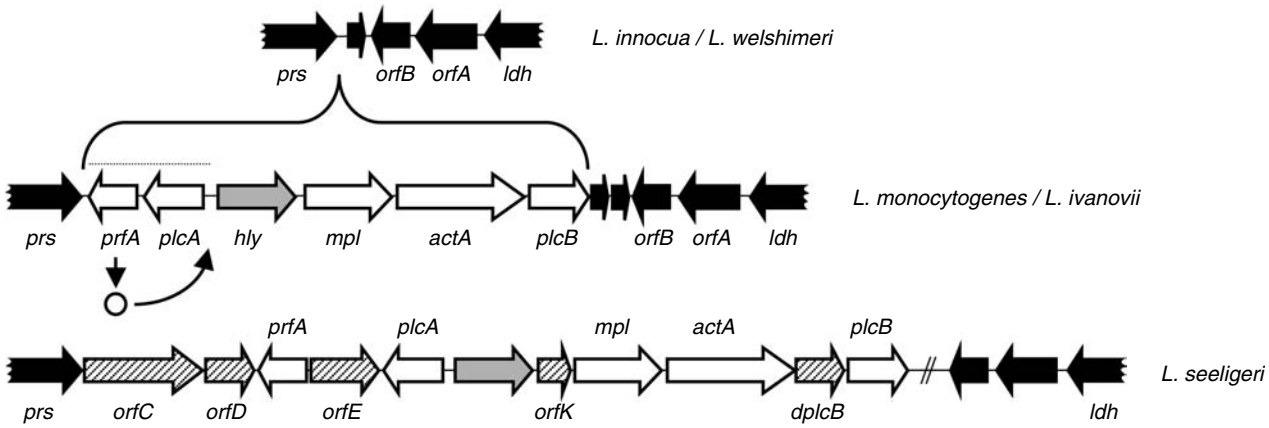



**FIGURE 40.2** Genetic structure of LIPI-1 (central virulence gene cluster) and its insertion region in *Listeria* spp. Genes belonging to LIPI-1 are represented by empty arrows except *hly*, which is in grey. The LIPI-1 insertion region is delimited by the housekeeping loci *prs* (phosphoribosylpyrophosphate synthase) and *Idh* (lactate dehydrogenase). LIPI-1 is absent from the chromosomes of the non-pathogenic species, *L. innocua* and *L. welshimeri*, likely via deletion of the island. In the non-pathogenic species, *L. seeligeri*, LIPI-2 contains numerous insertions (hatched arrows). One of these insertions disrupts the *plcA-prfA* operon, inactivating a positive autoregulatory loop required for the synthesis of the central virulence regulator PrfA, PrfA activates the transcription of LIPI-1 genes and of other virulence determinants located elsewhere on the listerial chromosome. Modified from Vázquez-Boland *et al.* (2001a).

izing epitopes that do not inhibit binding to membranes map to domain 1 (Darji *et al*., 1996).

In the consensus model, initial membrane contact via domain 4 (Heuck *et al*., 2000; Ramacha¡ndran *et al*., 2002) would facilitate the interaction of toxin molecules with cholesterol. This would trigger a conformational change in the monomer, increasing its capacity to self-aggregate and, via the refolding of three α-helices of domain 3 into two membrane-spanning β-hairpins, its hydrophobicity and membrane affinity (Shatursky *et al*., 1999). By lateral diffusion, the vertically arranged membrane-bound monomers would collide with other monomers to form an oligomeric prepore (Shepard *et al*., 2000; Hotze *et al*., 2002; Heuck *et al*., 2003) via edge-to-edge association of domain 3 β-strands (Ramachandran *et al*., 2004). In a second step, the prepore would insert into the membrane by vertical collapse of the toxin structure, which would make the β-hairpins extend across the bilayer, forming a transmembrane β-barrel pore (Czajkowsky *et al*., 2004). The notion that membrane binding and disruption are dissociable events in CDC function is supported by experiments with LLO showing that monoclonal antibodies that neutralize cytolytic activity do not affect toxin binding to target membranes (Nato *et al*., 1991; Darji *et al*., 1996). This is possible if these antibodies block downstream events of toxin fuction, such as oligomerization or pore formation.

## Domain 4

Most targeted mutagenesis studies have focused on domain 4, as this region contains the conserved C-terminal Trp-rich motif, ECTGLAWEWWR, a hallmark of this toxin family. As in other CDCs (Pinkney *et al*., 1989; Saunders *et al*., 1989; Sekino-Suzuki *et al*., 1996; Baba *et al*., 2001), mutational analyses in LLO have shown that this domain, in particular the conserved motif and the C-terminus, is critical for activity (Mengaud *et al*., 1988; Kohda *et al*., 2002). In LLO, single amino-acid substitutions in the undecapeptide resulted in *L. monocytogenes* strains with various degrees of attenuation proportional to the loss of cytolytic activity (Michel *et al*., 1990). LSO from the non-pathogenic *L. seeligeri* carries a naturally occurring Ala-to-Phe substitution in the undecapeptide (Haas *et al*., 1992) (Figure 40.1), which has been shown to be responsible for the weak cytolytic activity for sheep erythrocytes exhibited by this toxin (Ito *et al*., 2001).

### *Role of the single Cys residue*

The undecapeptide motif contains the Cys residue that mediates the characteristic reversible inhibition by oxidation—and reactivation by thiol-reducing agents—that gave this toxin family its former name, "oxygen-labile" or "thiol-activated" hemolysins. The activating effect of thiol-reducing agents was initially thought to be due to the breakage of an intramolecular bond, but sequencing of the LLO gene and of other CDC genes revealed that in most cases only one Cys residue is present in the toxin molecule. This residue was also thought to be essential for activity and to be involved in cholesterol binding (see below) (Smyth and Duncan, 1978; Alouf and Geoffroy, 1991; Alouf, 1999). However, although replacement of the Cys residue by Ala in LLO rendered the toxin insensitive to


**FIGURE 40.3** Theoretical three-dimensional structure of the soluble LLO monomer based on the crystal structure of PFO. Domains are indicated by numbers (the arrow points to the Trp-rich undecapeptide). Ribbon model generated using PyMOL 0.97 (DeLano, 2004).

inactivation by oxidation or thiol-alkylating agents, it had no significant effect on cytolytic activity (Michel *et al*., 1990). Similar results were obtained with other toxins of the family (Pinkney *et al*., 1989; Saunders *et al*., 1989).

The non-essential role of the Cys residue raises questions as to why it is subject to such pressure for conservation. In LLO and other CDCs, its replacement by bulkier residues abolishes most of the activity, suggesting that the mechanism underlying the Cys-mediated reversible inhibition of cytolytic activity involves steric hindrance (Pinkney *et al*., 1989; Saunders *et al*., 1989; Michel *et al*., 1990). Such an effect may result

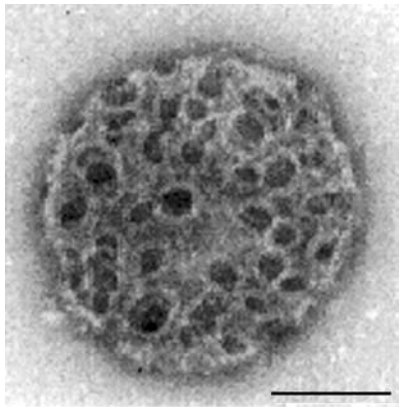


**FIGURE 40.4** Ring-shaped pores formed by LLO on a sheep erythrocyte ghost. Bar indicates 100 nm. Reproduced from Jacobs *et al.* (1998), with permission.

from the formation of toxin dimers or heterodimers with other proteins via disulfide bridges. Indeed, ILO copurifies with a major 27-kDa protein of the *L. ivanovii* culture supernatant, and this heterodimer is resolved by treatment with thiol-reducing agents (Vázquez-Boland *et al*., 1989a). This 27-kDa protein is another listerial virulence factor, the small/excreted internalin i-InlE (Engelbrecht *et al*., 1998), the mature form of which contains a single Cys. This suggests that the Cys residue present in the undecapeptide of most CDCs may play a role in the targeting of other virulence-associated proteins to the eukaryotic cell membrane. It may also have a regulatory role *in vivo*, for example, by enhancing toxin activity in low-pH (reducing) environments, such as the acidified vacuolar compartment.

### *Cholesterol binding*

Based on the capacity of cholesterol to inhibit the cytolytic activity of CDCs irreversibly and the fact that these toxins are active only against cholesterol-containing membranes, it is generally accepted that this membrane lipid acts as the toxin receptor (Smyth and Duncan, 1978; Alouf and Geoffroy, 1991). Indeed, many studies have shown that the extent of CDC interaction with the membrane is directly dependent on the amount of cholesterol present on that membrane (Jacobs *et al*., 2001; Heuck *et al*., 2000; Waheed *et al*., 2001), and a cholesterol-binding region has been unambiguously traced to domain 4 in PFO (Shimada *et al*., 1999, 2002). Consistent with this, C-terminal truncations in LLO, whether involving the undecapeptide or not, abolished binding to immobilized cholesterol (Kohda *et al*., 2002). Experiments with recombinant PLY polypeptides also showed that domain 4 alone binds free cholesterol, whereas a truncated protein

comprising only domains 1–3 does not (Baba *et al*., 2001). In some of these studies, the abolition of cholesterol-binding activity was associated with a loss of membrane-binding activity (Shimada *et al*., 1999; Baba *et al*., 2001), in line with the view that cholesterol recognition is essential for initial interaction with the membrane.

However, experiments in which preincubation of LLO with cholesterol did not impede the binding of the toxin-cholesterol complexes to the cell membrane (Jacobs *et al*., 1998) challenged the above notion. A similar dissociation of cholesterol- and membrane-binding activities was observed in experiments using PFO mutants with single Phe substitutions in the three Trp residues of the undecapeptide. These mutations impaired membrane binding, but did not affect cholesterol binding (Sekino-Suzuki *et al*., 1996). The observation that CDCs in solution form highly stable amphiphilic complexes with free cholesterol led to the development of a simple one-step purification method by selective precipitation with cholesterol (Vázquez-Boland *et al*., 1989a). Using this technique, an inactive, truncated LLO lacking the 49 C-terminal residues (i.e., approx. half of domain 4, including the entire conserved undecapeptide) (Mengaud *et al*., 1988) was shown to form cholesterol-toxin complexes (Vázquez-Boland *et al*., 1989b). Thus, domains involved in cholesterol binding appear to be present outside the undecapeptide and C-terminus of the toxin, either in the remaining part of domain 4 (a possibility not supported by recent evidence obtained with fluorescent probes showing that, except for its tip—the Trp-rich loop—all parts of domain 4 in membrane-bound PFO are accessible to the solvent; Heuck *et al*., 2000) or in the N-terminal region. The latter option is consistent with the observation that although the cholesterol-complexed LLO binds to target membranes, there is no oligomerization (Jacobs *et al*., 1998), suggesting that preincubation with cholesterol blocks sites involved in pore formation (which reside in the N-terminal region; see above). Moreover, the above-mentioned PFO mutants that showed reduced membrane-binding and cytolytic capacities while retaining cholesterol-binding activity were still able to form ring-shaped structures on membranes (Sekino-Suzuki *et al*., 1996). These observations suggest that, rather than acting as the toxin receptor, cholesterol might play a critical role in subsequent stages of membrane-toxin interaction, i.e., during oligomerization and/or membrane insertion.

The view that cholesterol's primary role is to facilitate membrane insertion has received strong support from recent studies showing that cholesterol-depleted membranes stalled PFO, SLO, and intermedilysin (ILY, from *Streptococcus intermedius*) at the prepore stage. All three toxins regained lytic activity if cholesterol was restored to the membrane, suggesting that this lipid is critically involved in the prepore to transmembrane pore transition (Giddings *et al*., 2003).

### *Role of the conserved undecapeptide*

The conserved ECTGLAWEWWR undecapeptide forms a hydrophobic loop at the base of domain 4 (Figure 40.3). This structure penetrates the lipid bilayer and is thought to be responsible for the initial attachment of the CDC monomers to the target membrane during prepore formation (Heuck *et al*., 2000; Ramachandran *et al*., 2002). However, its exact role in toxin function remains unclear, as the contradictory data obtained with LLO illustrate. Thus, membrane binding was not affected in LLO mutants with impaired cytolytic activity due to amino-acid replacements in the undecapeptide, including Trp to Ala substitutions, which almost totally abolished toxin function (Michel *et al*., 1990). Similarly, monoclonal antibodies produced against a synthetic LLO undecapeptide and that recognized the immobilized toxin did not inhibit membrane binding or cytolysis (Nato *et al*., 1991). In contrast, a neutralizing monoclonal antibody raised against native toxin and that specifically bound to the undecapeptide caused LLO membrane-binding inhibition and no longer recognized its target epitope in a pre-formed toxin/ membrane complex (Jacobs *et al*., 1999). The latter experiments are consistent with results obtained with the above-mentioned weakly hemolytic PFO mutants containing Phe-to-Trp substitutions in the undecapeptide, as these displayed significantly impaired erythrocyte membrane-binding activities (Sekino-Suzuki *et al*., 1996). Circular dichroism analyses of membrane-associated mutant PFO toxins suggested that both Trp438 and Trp439 were involved in a conformational change occurring during pore formation (Nakamura *et al*., 1998). Thus, the impaired interaction with the membrane seen with the PFO mutants or LLO bound to the undecapeptide-specific antibody may indicate a role for the conserved motif in the adoption of the correct folding required for membrane insertion and pore formation, rather than a role in the initial stages of toxin-membrane interaction. Such an interpretation is consistent with the observed inhibition of PLY self-interaction by derivatization of the single Cys residue with the thiol-active agent dithio (bis)nitrobenzoic acid (Gilbert *et al*., 1998).

Recently, PFO and ILY mutants displaying a significant loss of hemolytic activity, but retaining wild-type levels of membrane-binding activity, have been shown to oligomerize on the membrane but to be unable to form a pore complex, consistent with a role for the undecapeptide in the prepore-to-pore conversion.

This study also showed that target cell recognition by ILY depends on domain 4, but does not involve the undecapeptide (Polekhina *et al*., 2005) (see below).

### Domains 1–3

A recent study with independently expressed N-terminal (domains 1–3) and C-terminal (domain 4) LLO polypeptides has shown that the two protein fragments were able to bind to erythrocyte membranes and intact mammalian cells, the former even with greater affinity (Dubail *et al*., 2001). Intriguingly, functional complementation was reported to occur after coproduction of both LLO subfragments in *L. monocytogenes*, suggesting that they can reassemble to form a pore complex (Dubail *et al*., 2001). Also intriguing was that a Δ*hly L. monocytogenes* strain, *trans*-complemented with a plasmid encoding a truncated toxin variant lacking the first three residues of the native protein, displayed similar levels of hemolytic activity to wild-type *L. monocytogenes in vitro*, but apparently disrupted the phagocytic vacuole less efficiently and was two orders of magnitude less virulent in the mouse model (Lety *et al*., 2003). It is unclear whether this was actually due to the lack of these three first residues in LLO, which would imply that they play a specific role in the interaction with the phagosomal membrane, or to problems of *in vivo* instability of the plasmid construct encoding the truncated LLO toxin.

## ROLE IN INTRACELLULAR PARASITISM

### Cell biology of *Listeria* infection

*Listeria* belong to a subgroup of intracellular bacterial pathogens that replicate in the cytosol and not in a membrane-bound vacuole. Soon after phagocytosis, whether by macrophages or by any other susceptible cell type actively invaded by these bacteria (epithelial cells, hepatocytes, endothelial cells, etc.), listeriae are seen free in the cytosol. Intracytosolic bacteria polymerize host cell actin at one of their poles by means of the surface protein ActA, a mechanism that propels them across the cytosol at a speed of 0.3 μm/s. The movement is random, and some bacteria eventually reach the cell periphery and protrude from the cell surface at the tip of pseudopod-like evaginations, which penetrate into neighboring cells. This triggers a phagocytosis process, resulting in the formation of a "secondary" phagosome that is surrounded by a double membrane, from which *Listeria* escape again to begin a new replication cycle (Figure 40.5). Thanks to this direct cell-to-cell spread mechanism, *Listeria* rarely leave the intracellular host compartment, facilitating avoidance of the humoral effectors of the immune system and phagocytosis by migrant neutrophils. By rapidly spreading to neighboring cells, they escape the cellular immune response typically directed against infected cells displaying cytosol-released bacterial epitopes in association with MHC class I molecules (San Mateo *et al*., 2002). This strategy contrasts with that used by many other intracellular pathogens (e.g., *Salmonella* or *Mycobacterium*), which avoid the cellular immune response by hiding inside a vacuole, an enclosed environment that prevents/delays the presentation of antigens via the cytosolic MHC class I pathway (for recent reviews see Vázquez-Boland *et al*., 2001b; Portnoy *et al*., 2002; Dussurget *et al*., 2004). Efficient disruption of the phagocytic vacuole membranes is therefore a key process in *Listeria* pathogenesis.

### Role of LLO in phagosome disruption

The pathogenic *Listeria* spp, *L. monocytogenes* and *L. ivanovii*, are hemolytic, whereas the non-pathogenic spp, *L. innocua*, *L. welshimeri*, and *L. grayi*, are non-hemolytic. The only exception to this rule is *L. seeligeri*, which is hemolytic but non-pathogenic. However, as mentioned above, the hemolysin from this species, LSO, is weakly active and is produced in minute amounts (see below). The strong correlation between hemolytic activity and pathogenicity in the genus *Listeria* led various groups to generate, in the mid-late 1980s, isogenic hemolysin mutants by transposon mutagenesis. Analysis of the insertion region in one of these mutants led to the identifica-

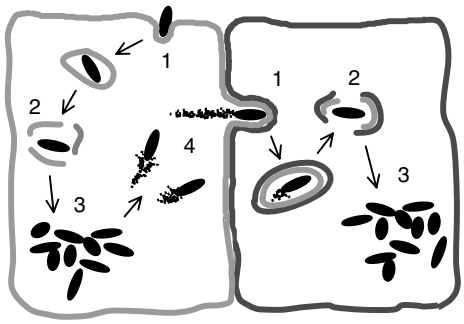



**FIGURE 40.5** Schematic representation of the intracellular infection cycle of pathogenic *Listeria*. PrfA-dependent virulence factors acting at each of the steps of the cycle: (1) cell invasion: surface proteins InlA, InlB and ActA; (2) escape from the phagosome: LLO assisted by the phospholipases, PlcA and PlcB (plus the SmcL sphingomyelinase in *L. ivanoii*); (3) rapid intracellular replication: hexose phosphate transporter, Hpt; (4) actin-based motility and cell-to-cell spread: the actin-polymerising protein, ActA.

tion and characterization of the *hly* gene (Mengaud *et al*., 1987, 1988). The non-hemolytic mutants were much less virulent in mice (increase greater than 4 logs in $LD_{50}$), their spontaneous revertants recovered full pathogenicity, and their *trans*-complementation with the *hly* gene restored virulence to wild-type levels (Gaillard *et al*., 1986; Kathariou *et al*., 1987; Portnoy *et al*., 1988; Cossart *et al*., 1989). Cell culture–based infection assays with these mutants showed that LLO is required for the intracellular survival and proliferation of *L. monocytogenes* in macrophages and non-professional phagocytes (Gaillard *et al*., 1987; Kuhn *et al*., 1988; Portnoy *et al*., 1988). Electron microscopy of infected cells revealed that *hly* mutants remained entrapped within phagosomes, indicating that LLO mediates disruption of the phagosome membrane (Gaillard *et al*., 1987). This was elegantly confirmed by experiments in which the *hly* gene was expressed in *Bacillus subtilis*; this conferred on the non-pathogenic bacterium the ability to escape from the phagocytic vacuole and to replicate within host cells (Bielecki *et al*., 1990). LLO not only mediates lysis of the primary phagosomes formed after the uptake of extracellular bacteria, but it is also required for the efficient escape of *L. monocytogenes* from the double-membrane secondary vacuole that forms upon cell-to-cell spread (Gedde *et al*., 2000; Dancz *et al*., 2002). Although LLO plays a critical role in phagosome disruption, three other membrane-damaging listerial proteins, the phospholipases C PlcA and PlcB, and the sphingomyelinase SmcL in *L. ivanovii*, cooperate in this process (see Vázquez-Boland *et al*., 2001b). The membrane lesions caused by LLO (Figure 40.4) probably facilitate the access of these enzymes to their substrates in the phagosomal membrane, leading to its total dissolution.

The CDC from *L. ivanovii*, ILO, plays a similar role in intracellular infection. Thus, like *L. monocytogenes* $LLO^-$ mutants, *L. ivanovii* $ILO^-$ mutants are strongly impaired in intracellular growth (Figure 40.6), and expression of the ILO gene in a Δ*hly* mutant of *L. monocytogenes* complements the absence of LLO (Frehel *et al*., 2003). Even LSO from the non-pathogenic species *L. seeligeri*, albeit weakly active, appears to be able to promote phagosome disruption. The weakly hemolytic phenotype of this species is in part due to defective expression of the virulence gene (*hly*)-activator protein PrfA as a result of the insertion of a divergently transcribed ORF between the two genes of the *plcA-prfA* operon (Figure 40.2). Complementation of *L. seeligeri* with a functional copy of this operon activated the expression of LSO and conferred to the bacterium the ability to escape from the phagosome and to replicate in the cytosol (Karunasagar *et al*., 1997).

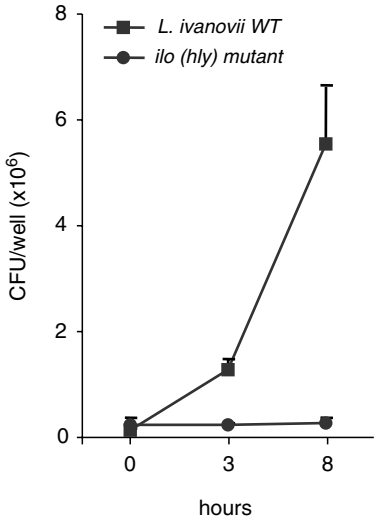



**FIGURE 40.6** Intracellular growth of wild-type *L. ivanovii* and an isogenic $ILO^-$ mutant in bovine MDBK cells. As with *L. monocytogenes*, inactivation of the *hly* gene results in total loss of intracellular proliferation capacity in *L. ivanovii*.

## Adaptation of LLO to the intracellular niche

Experiments in which the genes encoding SLO and PFO were expressed in *B. subtilis* showed that, despite their very close structural and functional similarities, not any CDC can supplant LLO's role in intracellular parasitism. SLO did not facilitate intracellular growth, whereas PFO did; however, in contrast to what is observed with LLO, PFO-mediated growth was associated with host cell damage (Portnoy *et al*., 1992). Expression of the *pfo* gene also failed to restore virulence for mice in a *L. monocytogenes hly* mutant. The resulting strain was hemolytic and showed some ability to escape from phagosomes, but PFO damaged the host cells, thereby preventing normal intracellular growth (Jones and Portnoy, 1994). These results indicated that LLO, produced by an intracellular pathogen requiring an intact intracellular niche to replicate and to spread in host tissues, has evolved mechanisms for preventing cytoxicity not found in PFO, originating from an extracellular pathogen associated with infections characterized by massive tissue destruction. PFO mutations resulting in a lack of cytotoxicity were identified by selecting for *pfo*-expressing *L. monocytogenes* mutants capable of normal intracellular growth. One type of mutation shifted the pH optimum of PFO (normally active in both acidic and neutral conditions) to a value similar to that for LLO, whereas the other decreased the cytosolic half-life of the toxin, providing

insight into the mechanisms by which LLO-mediated cytotoxicity is controlled (Jones *et al*., 1996).

Indeed, weak activity at pH 7.4 (similar to that of the eukaryotic cell cytosol) and a pH optimum of 5.5 (similar to that within phagosomes) is a clever strategy to restrict the membrane-damaging activity of LLO to the acidified vacuole, and thereby to prevent potential deleterious effects of toxin leakage into the cytosol. Evidence for such phagosome-specific compartmentalized activity of LLO has been provided by experiments using the membrane-impermeant fluorophore, 8-hydroxypyrene-1,3,6-trisulfonic acid, in murine macrophages. Rapid acidification of *L. monocytogenes*-containing phagosomes was observed, followed by an increase in pH and the release of the dye from the vacuole. Loss of the fluorophore was prevented by lysosomotropic agents, such as ammonium chloride and bafilomycin A1 (Beauregard *et al*., 1997). These data are consistent with activation of LLO in the acidified phagosome sequentially leading to membrane permeabilization, pH equilibration, and the automatic deactivation of LLO. A single amino acid in domain 4, Leu461 (position 438 in the mature protein as shown in Figure 40.1), was identified as being responsible for the low optimum pH of LLO. Among CDCs, only listerial hemolysins have a Leu residue at this position. The replacement of Leu461 by Thr, as in PFO, caused a 10-fold increase in LLO activity at neutral pH, and *L. monocytogenes* carrying the mutant LLO toxin were cytotoxic (Glomski *et al*., 2002).

The short half-life of LLO in the cytosol is due to the presence in the N-terminus of a PEST-like sequence, normally involved in the targeting of eukaryotic proteins for cytosolic degradation. Deletion of this sequence from LLO increased cytotoxicity and lowered the virulence of the strain; conversely, introduction of the PEST sequence into PFO reduced cytotoxicity, and the mutant PFO toxin supported intracellular growth when produced in *L. monocytogenes* (Decatur and Portnoy, 2000).

The relevance of these distinctive features of LLO to pathogenesis is illustrated by a recent study in mice using *L. monocytogenes* strains with mutations that fail to compartmentalize toxin activity. The more cytotoxic the strain in cell culture, the less virulent it was for mice. The depletion of neutrophils, primarily involved in the killing of extracellular *Listeria*, and treatment with gentamicin, active only against extracellular bacteria, increased the relative virulence of the cytotoxic strain, reflecting that the control of LLO cytolytic activity is a key element in the avoidance of extracellular host defenses by *L. monocytogenes* (Glomski *et al*., 2003).

## OTHER ROLES IN INFECTION

### Immune response

LLO is critically involved in the immune response to *L. monocytogenes* in several ways: (i) The LLO-mediated release of bacteria into the cytosol and subsequent intracellular growth are essential for MHC class I-restricted listerial antigen presentation and the induction of specific protective cytotoxic CD8+ T cells (Berche *et al*., 1987; Brunt *et al*., 1990; Barry *et al*., 1992; Hiltbold *et al*., 1996). It has recently been reported that cytosolic listeriae activate type I IFN expression and many IFN-β-induced genes, whereas phagosome escape-deficient (*hly*) mutants do not (Stockinger *et al*., 2002; McCaffrey *et al*., 2004). Non-pathogenic bacteria engineered to enter the host cytosol via heterologous *hly* expression also triggered this response (O'Riordan *et al*., 2002). Thus, via its membrane-disrupting activity, LLO may play a crucial role not only in the development of the acquired immune response, but possibly also in the activation of key innate immunity mechanisms. (ii) LLO is itself a major protective antigen recognized by *Listeria*-specific $CD8^+$ cytotoxic T cells (CTLs) during infection (Berche *et al*., 1987a; Safley *et al*., 1991; Bouwer *et al*., 1992; Hess *et al*., 1996; Sirard *et al*., 1997). LLO is processed very efficiently into peptides that are presented by MHC class I molecules (Villanueva *et al*., 1995), and one such peptide, nonamer LLO 91–99, is an immunodominant epitope that induces specific $CD8^+$ CTLs, which protect *in vivo* against *L. monocytogenes* infection and confer significant anti-*Listeria* immunity on naive mice upon passive transfer (Pamer *et al*., 1991; Harty *et al*., 1992). (iii) Pore formation by exogenous LLO has been shown to mediate the delivery of soluble antigens to the TAP-dependent cytosolic MHC class I antigen-presentation pathway (Darji *et al*., 1995, 1997), providing an additional mechanism for the generation of $CD8^+$ CTLs against antigens secreted/released by extracellular *L. monocytogenes*. (iv) LLO elicits a potent humoral response and anti-LLO antibodies are detectable after infection with *L. monocytogenes* (see below). Administration of a murine anti-LLO neutralizing monoclonal antibody increased resistance to *Listeria* infection in mice (Edelson *et al*., 1999), thus challenging the old paradigm of cell-mediated immunity, according to which antibodies are not involved in protection against intracellular parasites (Kaufmann, 1993). It is therefore possible that the humoral arm of the immune system contributes to protective immunity against *L. monocytogenes* and that LLO also plays a key role in this protection. Finally, (v) through its modulin activity,

LLO may influence orchestration of the immune response to *Listeria* (see next section).

## Modulation of host responses

LLO has been shown to induce a number of host-cell responses in a variety of cell types, indicating that this toxin is a versatile virulence factor, not only having a direct physical action on phagosome membranes, but also signaling effects on host cells that can influence the fate of *Listeria* infection. One of the mechanisms possibly involved is related to the ability of exogenous LLO to permeabilize cell membranes. Exogenous LLO at sublytic concentrations has been shown to produce $Ca^{2+}$-permeable pores, causing the influx of extracellular $Ca^{2+}$ions into the cell (Repp *et al*., 2002). This may enable cell surface-attached *L. monocytogenes* bacteria to modulate/enhance their entry into host cells, the key first step in listerial intracellular parasitism (Dramsi and Cossart, 2003). LLO-mediated permeabilization of the cell membrane is also required for the induction by the listerial phosphatidylinositol-specific phospholipase C (PlcA) of phosphoinositide hydrolysis and diacylglycerol (DAG) accumulation in human endothelial cells (Sibelius *et al*., 1996a), and for lipid mediator generation and activation in neutrophils (Sibelius *et al*., 1999). Infection experiments with isogenic *hly* and *plcA L. monocytogenes* mutants have also suggested that LLO is required for the induction of PlcA-mediated DAG generation and PKC translocation early in macrophage infection (Wadsworth and Goldfine, 2002). CDC-mediated translocation of effector proteins has recently been demonstrated with the related streptococcal toxin SLO, and it has been suggested that this mechanism may play in Gram-positive bacteria a role similar to that of the type III secretion systems in Gram-negative bacteria (Madden *et al*., 2001; Walev *et al*., 2001).

LLO may also act as a modulin via a mechanism independent of its membrane-permeabilizing activity. Thus, exposure to non-cytolytic LLO-cholesterol complexes has been shown to induce cytokine gene expression (Nishibori *et al*., 1996) and IL-1 release (Yoshikawa *et al*., 1993) in murine macrophages, the release of IL-12, IL-18, and IFN-γ in murine spleen cells (Kohda *et al*., 2002; Nomura *et al*., 2002; Ito *et al*., 2003), and the induction of lipid second messengers in human endothelial cells (Sibelius *et al*., 1996b). Non-cytolytic LLO-cholesterol complexes retain the capacity to bind to cell membranes (see above), and such responses are unlikely to be generated unless the toxin interacts with cell surface receptors, suggesting that CDCs are able to recognize membrane components other than cholesterol. Indeed, it has recently been shown that the human cell-specific CDC ILY from *S. intermedius* uses CD59 as its cellular receptor (Giddings *et al*., 2004). These observations raise the interesting possibility that LLO released extracellularly and rendered non-cytolytic by complexation with free cholesterol present in body fluids may be targeted to host cell surfaces and mediate signaling events upon binding to specific membrane receptors.

Infection experiments in cell culture models using wild-type and *hly*-mutant *L. monocytogenes* bacteria suggest that intracellularly delivered LLO may also induce signaling events leading to a variety of cell responses. These include activation of the Raf-Mek-MAP kinase pathway in epithelial cells (Tang *et al*., 1994, 1996; Weiglein *et al*., 1997), the induction of mucus exocytosis in intestinal cells (Coconnier *et al*., 1998), cytokine gene expression in macrophages (Kuhn and Goebel, 1994; Nishibori *et al*., 1996), degranulation and leukotriene formation in neutrophils (Sibelius *et al*., 1999), and Fas ligand expression on T lymphocytes (Zenewicz *et al*., 2004). In endothelial cells—a major target cell in the neuropathogenesis and placental invasion by *L. monocytogenes*—LLO provokes lipid mediator generation (phosphoinositides, DAG and ceramide) (Sibelius *et al*., 1996a), the up-regulation of adhesion molecules (Drevets, 1998; Kayal *et al*., 1999; Krüll *et al*., 1997), nitric oxide (NO) synthesis, and the release of proinflammatory cytokines such as IL-6, IL-8, monocyte chemotactic protein-1, and granulocyte-macrophage colony-stimulating factor (Kayal *et al*., 1999; Rose *et al*., 2001). This is associated with the induction of the NF-κB signaling pathway via activation of the IκB kinase β subunit of the IKK complex, an effect that has been shown to be mediated by LLO in experiments involving heterologous expression of the *hly* gene in the non-pathogenic species *L. innocua* (Kayal *et al*., 1999, 2002).

Further evidence that LLO is important in modulating the immune response comes from experiments using purified LLO (Carrero *et al*., 2004a), which showed that the toxin was responsible for lymphocyte apoptosis as typically seen in mouse splenic cells both *ex vivo*, especially in type I IFN-activated lymphocytes (Carrero *et al*., 2004b), and *in vivo* (Merrick *et al*., 1997), upon infection with *L. monocytogenes*. The massive regression of the spleen white pulp appears to be pathognomonic of *Listeria* infection in mice (Marco *et al*., 1991; Conlan, 1996) and may well be caused by LLO-mediated apoptotic lymphocyte depletion. Thus, LLO appears to play dual immunomodulatory roles with similar outcomes in host-parasite interaction, inducing the mobilization of innate host defenses (thereby favoring the spread of infection by making available more host cells for bacterial replication) and at the same time attenuating the T cell-mediated responses it inevitably triggers through its role as

immunodominant, cytosolic-released antigen. This immunosuppressive effect is consistent with other observations linking LLO to the down-regulation of major histocompatibility complex I (antigen I-Ab) and II (antigen H-2K$^d$) gene expression, as observed in infected murine macrophages (Schüller *et al*., 1998), and the interference with antigen presentation through the induction of apoptosis in dendritic cells (Guzmán *et al*., 1996) and type I IFN-sensitized macrophages (Stockinger *et al*., 2002).

A note of caution must be introduced, however, as although in most cases the above signaling effects can be reproduced by exposure to exogenous purified LLO, it remains unclear whether such effects are directly exerted by the toxin, are due to other bacterial components (which may also be present as contaminants in the toxin preparations), or are just indirect, nonspecific consequences of membrane destabilization or bacterial intrusion into the cytosol.

Finally, a recent study has revealed a facet of LLO that may be highly relevant to pathogenesis: lipid raft clustering. Gekara and Weiss (2004) have shown that the treatment of J774 murine macrophages with LLO led to raft aggregation and clustering of CD14 and CD24 receptors, suggesting that this may be a key mechanism by which the listerial toxin (and potentially any other CDC) modulates host cell responses.

## APPLICATIONS OF LLO RESEARCH

With its optimum activity at pH 5.6, its reduced activity at neutral pH, and its short cytosolic half-life, LLO is an ideal molecule for selective perforation of the phagosomal membrane without collateral damage to the host cell. This unique property of LLO has been exploited to develop strategies to introduce therapeutic macromolecules into the cytosol. Needless to say, since the discovery of its key role as the immunodominant CD8$^+$ antigen in the protective immune response against *L. monocytogenes* in the mouse model, LLO has been extensively used as a research tool in basic immunological studies (Harty and Bevan, 1992; Unanue, 1997; Shen *et al*., 1998) and in investigations on the efficacy of experimental vaccines (Cornell *et al*., 1999; Rüssmann *et al*., 2001, 2003; Yoshida *et al*., 2001; Barry *et al*., 2003).

### Bacteria-based antigen and DNA delivery vehicles

A number of bacteria have been engineered to induce a robust CD8$^+$-based cytotoxic immune response, necessary for the clearance of intracellular pathogens and tumors, via heterologous expression of the listerial *hly* gene alone or in combination with recombinant antigens. Examples include *Escherichia coli* (Hu *et al*., 2004; Radford *et al*., 2003), *Mycobacterium bovis* BCG (Hess *et al*., 1998), or avirulent mutants of *Salmonella enterica* serovar Typhimurium (Hess *et al*., 2000) and *Bacillus anthracis* (Sirard *et al*., 1997), all of which have provided ample proof of the principle that LLO can indeed mediate the efficient presentation of antigens via the cytosolic MHC class I-restricted pathway (Dietrich *et al*., 2001).

The considerable amount of knowledge gathered on the pathogenesis of *Listeria* infection and its molecular determinants make *L. monocytogenes* bacteria themselves an ideal platform for the rational development of recombinant oral vaccines based on LLO-mediated induction of cellular immunity (Iknomidis *et al*., 1995; Guzman *et al*., 1997; Weiskirch and Paterson, 1997; Dietrich *et al*., 2003). A *Listeria*-based antigen carrier system has the clear advantage that its gene regulation machinery has naturally evolved to ensure the correct spatio-temporal synthesis of LLO (Kreft and Vázquez-Boland, 2001). Being a Gram-positive bacterium, *L. monocytogenes* is also devoid of lipopolysaccharide-associated toxicity. Moreover, in addition to CD8$^+$ CTLs, *L. monocytogenes* also elicits a vigorous Th1-

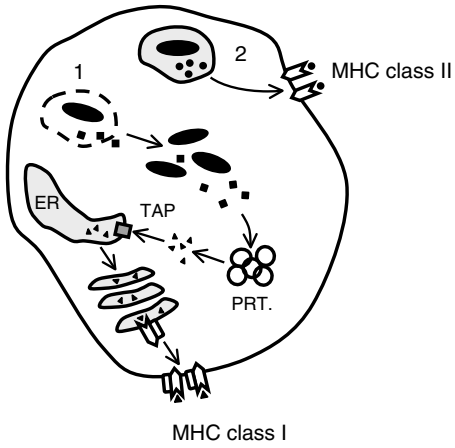



**FIGURE 40.7** Bacteria-based intracellular vaccine delivery and antigen processing and presentation pathways involved. (1) LLO-expressing bacteria lyse the phagosome and their antigens (squares) leak into the cytosol, where they are processed by the proteasome (PRT) into peptides (triangles); these peptides are translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing and presentation (TAP) and loaded onto major histocompatibility complex (MHC) class I molecules, leading to induction of a CD8$^+$-mediated response. (2) In contrast, bacteria that remain entrapped within phagosomes present their antigens (circles) primarily via the endosomal, MHC class II pathway, leading to induction of a CD4$^+$-mediated immune response.

biaised $CD4^+$ T-cell response (Guzman *et al*., 1998), possibly because a portion of the bacteria remain entrapped within phagosomes and, consequently, their antigens are processed through the endosomal/phagolysosomal pathway (Figure 40.7). Combined deletions in known virulence genes, such as the *actA-plcB* operon required for cell-to-cell spread, and in metabolic genes leading to auxotrophy—e.g., genes of the D-alanine biosynthetic pathway involved in the synthesis of the cell wall—can be used to create hyperattenuated, environmentally safe *Listeria* vaccine carriers (Thompson *et al*., 1998; Angelakopoulos *et al*., 2002). Robust cell-mediated immunity has been achieved using recombinant *L. monocytogenes* as a vehicle for expression of genes encoding a variety of proteins, including viral (Ikonomidis *et al*., 1994, 1997; Frankel *et al*., 1995; Shen *et al*., 1995) and tumor (Pan *et al*., 1995; Jensen *et al*., 1997; Paglia *et al*., 1997) antigens. Promising results have been obtained in mice with *Listeria*-based anti-HIV (Mata *et al*., 1999, 2001; Lieberman and Frankel, 2003; Peters *et al*., 2003) and anti-cancer (Pan *et al*., 1995; Gunn *et al*., 2001) vaccines. Protection and even the regression of established tumors have been achieved with the latter, demonstrating the therapeutic potential of this bacterial carrier and the LLO-mediated antigen delivery system for curing neoplastic diseases.

*L. monocytogenes* can also be used as a vector for transferring DNA into eukaryotic cells. A plasmid inserted with model antigen genes under the control of a eukaryotic promoter and a *Listeria* phage endolysin gene under the control of the strictly PrfA-dependent *actA* gene promoter (which is selectively activated in the cytosol) were introduced into an *L. monocytogenes* strain attenuated by a deletion in the *actA-plcB* operon. Bacteria underwent lysis upon entry into the cytosol and generated an MHC class I–restricted immune response against the plasmid-encoded model antigens (Dietrich *et al*., 1998). These experiments demonstrated that *Listeria* also provides a system of potential value for the development of DNA vaccines and gene therapy tools (Spreng *et al*., 2000). DNA transfer to host cells has been achieved using LLO-expressing *E. coli* strains rendered invasive by introduction of the *Yersinia pseudotuberculosis inv* gene (Grillot-Courvalin *et al*., 1998).

## Other tools for the cytosolic delivery of macromolecules

Alternative strategies exploiting the capacity of LLO to deliver macromolecules to the cytosolic pathway include the co-administration of the toxin (Darji *et al*., 1995) or its co-encapsulation into pH-sensitive liposomes (Lee *et al*., 1996; Mandal *et al*., 2003). Efficient MHC class I antigen presentation and $CD8^+$ responses have been achieved with these methods (Darji *et al*., 1997; Tanabe *et al*., 1999; Mandal and Lee, 2003), indicating that they are effective in rerouting the passenger or cargo proteins from the endosomal to the cytosolic antigen presentation pathways. LLO-liposomes (called "listeriosomes") can be used for bypassing the endosomal/lysosomal-mediated degradation and/or excretion pathway, thus ensuring that encapsulated chemotherapeutic drugs reach their intracellular targets, as recently shown with the plant-derived, antitumoral toxin gelonin (Provoda *et al*., 2003). Antisense oligonucleotides have also been successfully delivered into the cytosol using listeriosomes (Mathew *et al*., 2003).

## *In vivo* expression technology (IVET) systems

Based on the fact that *hly* mutants are non-hemolytic on blood agar plates and are significantly impaired in virulence, LLO has been used as both a reporter and a selection system to identify *in vivo*-expressed genes in *L. monocytogenes*. The system is based on the screening of *L. monocytogenes* chromosomal libraries generated by inserting random DNA fragments upstream of a promoterless *hly* gene cloned into a shuttle vector. These libraries are expressed in an Δ*hly* mutant, and selection is carried out in mice. Clones with increased survival are isolated, and those carrying constitutive or *in vivo*-induced promoters are differentiated via their respective $LLO^+$ and $LLO^-$ phenotypes. Among the *in vivo*-expressed genes identified was *plcA* (Gahan and Hill, 2000; Dubail *et al*., 2000), a known virulence determinant involved in intracellular survival, providing evidence of the workability of the LLO-based *in vivo* selection system.

## Diagnostic tools

LLO elicits a potent humoral response during infection (Grenningloh *et al*., 1998), and several studies have shown that the detection of LLO-specific antibodies can be used in the serodiagnosis of listeriosis and in serological surveys of *Listeria* infection both in humans (Berche *et al*., 1990; Gholizadeh *et al*., 1996, 1997) and animals (Miettinen and Husus, 1991; Low and Donachie, 1991; Low *et al*., 1992; L'hopital *et al*., 1993). The *hly* gene has been also used as target for the specific detection and quantification of *L. monocytogenes* by molecular (PCR-based) methods (Nogva *et al*., 2000; Rodríguez-Lázaro *et al*., 2004).

## ACKNOWLEDGMENTS

Although we have endeavored to cite all major contributions to current knowledge on LLO, due to space limitations or involuntary omission, some may not have been appropriately cited, for which we apologize to the authors. Our research on *Listeria* is funded by grants from The Wellcome Trust, the Spanish Ministry for Education and Science, and the European Union.

## REFERENCES

Alouf, J.E. (1999). Introduction to the family of the structurally related cholesterol-binding cytolysins ("sulfhydryl-activated") toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 2nd edit. (eds. J.E. Alouf and J.H. Freer), pp. 443–456. Academic Press, London.

Alouf, J.E. and Geoffroy, C. (1991). The family of the antigenically-related, cholesterol-binding "sulphydryl-activated" cytolytic toxins. In: *Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), pp. 149–186. Academic Press, London.

Angelakopoulos, H., Loock, K., Sisul, D.M., Jensen, E.R., Miller, J.F. and Hohmann, E.L. (2002). Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA/plcB* in adult volunteers: a dose escalation study of oral inoculation. *Infect. Immun.* **70,** 3592–3601.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2001). Essential role of domain 4 of pneumolysin from *Streptococcus pneumoniae* in cytolytic activity as determined by truncated proteins. *Biochem. Biophys. Res. Commun.* **281,** 37–44.

Barry, R.A., Bouwer, H.G.A., Clark, T.R., Cornell, K.A. and Hinrichs, D.J. (2003). Protection of interferon-gamma knockout mice against *Listeria monocytogenes* challenge following intramuscular immunization with DNA vaccines encoding listeriolysin O. *Vaccine*, **21,** 2122–2132.

Barry, R.A., Bouwer, H.G.A., Portnoy, D.A. and Hinrichs, D.J. (1992). Pathogenicity and immunogenicity of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immun.* **60,** 1625–1632.

Beauregard, K.E., Lee, K.D. Collier, R.J. and Swanson, J.A. (1997). pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes. J. Exp. Med.* **186,** 1159–1163.

Berche, P., Gaillard, J.-L., Geoffroy, C. and Alouf, J.E. (1987a). T cell recognition of listeriolysin O is induced during infection with *Listeria monocytogenes. J. Immunol.* **139,** 3813–3821.

Berche, P., Gaillard, J.-L. and Sansonetti, P. (1987b). Intracellular growth of *Listeria monocytogenes* as a prerequisite for *in vivo* induction of T cell-mediated immunity. *J. Immunol.* **138,** 2266–2271.

Berche, P., Reich, K.A., Bonnichon, M., Beretti, J.-L., Geoffroy, C., Ravenau, J., Cossart, P., Gaillard, J.-L., Geslin, P., Kreis, H. and Veron. M. (1990). Detection of anti-listeriolysin O for serodiagnosis of human listeriosis. *Lancet* **335,** 624–627.

Bhakdi, S. and Tranum-Jensen, J. (1987). Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Physiol. Biochem. Pharmacol.* **107,** 147–233.

Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin O. *Infect. Immun.* **47,** 52–60.

Bielecki, J. Youngman, P. Connelly, P. and Portnoy, D.A. (1990). *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. *Nature,* **345,** 175–176.

Bouwer, H.G.A., Nelson, C.S., Gibbins, B.L., Portnoy, D.A. and Hinrichs, D.J. (1992). Listeriolysin O is the target of the immune response to *Listeria monocytogenes*. *J. Exp. Med.* **175,** 1467–1471.

Brunt, L.M., Portnoy, D.A. and Unanue, E.R. (1990). Presentation of *Listeria monocytogenes* to CD8+ T cells requires secretion of hemolysin and intracellular bacterial growth. *J. Immunol.* **145,** 3540–3546.

Carrero, J.A., Calderon, B. and Unanue, E.R. (2004a). Listeriolysin O from *Listeria monocytogenes* is a lymphocyte apoptogenic molecule. J. Immunol. **172,** 4866–4874.

Carrero, J.A., Calderon, B. and Unanue, E.R. (2004b). Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to *Listeria* infection. *J. Exp. Med.* **200,** 535–540.

Coconnier, M.-H., Dlissi, E., Robard, M., Laboisse, C.L., Gaillard, J.-L. and Servin, A.L. (1998). *Listeria monocytogenes* stimulates mucus exocytosis in cultured human polarized mucosecreting intestinal cells through action of listeriolysin O. *Infect. Immun.* **66,** 3673–3681.

Conlan, J.W. (1996). Early pathogenesis of *Listeria monocytogenes* infection in the mouse spleen. *J. Med. Microbiol.* **44,** 295–302.

Cornell, K.A., Bouwer, H.G., Hinrichs, D.J. and Barry, R.A. (1999). Genetic immunization of mice against *Listeria monocytogenes* using plasmid DNA encoding listeriolysin O. *J. Immunol.* **163,** 322–329.

Cossart, P., Vincente, M.F., Mengaud, J., Baquero, F., Perez-Diaz, J.C. and Berche, P. (1989). Listeriolysin O is essential for virulence of *Listeria monocytogenes*: Direct evidence obtained by gene complementation. *Infect. Immun.* **57,** 3629–3636.

Czaikowsky, D.M., Hotze, E.M., Shao, Z., Tweten, R.K. (2004). Vertical collapse of a cytolysin prepore moves its transmembrane β-hairpins to the membrame. *EMBO J.* **23,** 3206–3215.

Dancz, C.E., Haraga, A., Portnoy, D.A. and Higgins, D.E. (2002). Inducible control of virulence gene expression in *Listeria monocytogenes*: temporal requirement of listeriolysin O during intracellular infection. *J Bacteriol.* **184,** 5935–5945.

Darji, A., Chakraborty, T., Wehland, J. and Weiss, S. (1995). Listeriolysin generates a route for the presentation of exogenous antigens by major histocompatibility complex class I. *Eur. J. Immunol.* **25,** 2967–2971.

Darji, A., Chakraborty, T., Wehland, J. and Weiss, S. (1997). TAP-dependent major histocompatibility complex class I presentation of soluble proteins using listeriolysin. *Eur. J. Immunol.* **27,** 1353–1359.

Darji, A., Niebuhr, K. Hense, M. Wehland, J. Chakraborty, T. and Weiss, S. (1996). Neutralizing monoclonal antibodies against listeriolysin: mapping of epitopes involved in pore formation. *Infect. Immun.* **64,** 2356–2358.

Darji, A., Stockinger, B., Wehland, J., Chakraborty, T. and Weiss, S. (1997). Antigen-specific T cell receptor antagonism by antigen-presenting cells treated with the hemolysin of *Listeria monocytogenes*: a novel type of immune escape. *Europ. J. Immun.* **27,** 1696–1703.

Decatur, A.L. and Portnoy, D.A. (2000). A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science*, **290,** 992–995.

DeLano, W.L. (2004). The PyMOL molecular graphics system. DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org.

De los Toyos, J.R., Méndez, F.J., Aparicio, J.F., Vázquez, F., García Suárez, M.M., Fleites, A., Hardisson, C., Morgan, P.J., Andrew, P.W. and Mitchell, T.J. (1996). Functional analysis of pneumolysin by use of monoclonal antibodies. *Infect. Immun.* **64,** 480–484.

Dietrich, G., Bubert, A., Gentschev, I., Sokolovic, Z., Simm, A., Catic, A., Kaufmann, S.H., Hess, J., Szalay, A.A. and Goebel, W. (1998). Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. *Nat. Biotechnol.* **16,** 181–185.

Dietrich, G., Hess, J., Gentschev, I., Knapp, B., Kaufmann, S.H. and Goebel, W. (2001). From evil to good: a cytolysin in vaccine development. *Trends Microbiol.* **9,** 23–28.

Dietrich, G., Viret, J.F. and Gentschev, I. (2003). Haemolysin A and listeriolysin—two vaccine delivery tools for the induction of cell-mediated immunity. *Int. J. Parasitol.* **33,** 495–505.

Dramsi, S. and Cossart, P. (2003).Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line. *Infect. Immun.* **71,** 3614–3618.

Drevets, D.A. (1998). *Listeria monocytogenes* virulence factors that stimulate endothelial cells. *Infect. Immun.* **66,** 232–238.

Dubail, I., Autret, N., Beretti, J.L., Kayal, S., Berche, P. and Charbit, A. (2001). Functional assembly of two membrane-binding domains in listeriolysin O, the cytolysin of *Listeria monocytogenes*. *Microbiology* **147,** 2679–2688.

Dubail, I., Berche, P. and Charbit, A. (2000). Listeriolysin O as a reporter to identify constitutive and in vivo-inducible promoters in the pathogen *Listeria monocytogenes*. *Infect. Immun.* **68,** 3242–3250.

Dussurget O., Pizarro-Cerda J., and Cossart P. (2004) Molecular determinants of *Listeria monocytogenes* virulence. Annu Rev *Microbiol* **58,** 587–610.

Edelson, B.T., Cossart, P., Unanue, E.R. (1999). Cutting edge: paradigm revisited: antibody provides resistance to *Listeria* infection. *J. Immunol.* **163,** 4087–4090.

Engelbrecht, F., Dominguez-Bernal, G., Dickneite, C. Hess, J., Greiffenberg, L. Lampidis, R., Raffelsbauer, D., Kaufmann, S.H.E., Kreft, J., Vazquez-Boland, J.-A. and Goebel, W. (1998). A novel PrfA-regulated chromosomal locus of *Listeria ivanovii* encoding two small, secreted internalins is essential for virulence in mice. *Mol. Microbiol.* **30,** 405–417.

Frankel, F.R., Hegde, S., Lieberman, J. and Paterson, Y. (1995). Induction of cell-mediated immune responses to human immunodeficiency virus type 1 Gag protein by using *Listeria monocytogenes* as a live vaccine vector. *J. Immunol.* **155,** 4775–4782.

Frehel, C., Lety, M.A., Autret, N., Beretti, J.L., Berche, P. and Charbit, A. (2003). Capacity of ivanolysin O to replace listeriolysin O in phagosomal escape and in vivo survival of *Listeria monocytogenes*. *Microbiology,* **149,** 611–620.

Gahan, C.G. and Hill, C. (2000). The use of listeriolysin to identify *in vivo* induced genes in the gram-positive intracellular pathogen *Listeria monocytogenes*. *Mol. Microbiol.* **36,** 498–507.

Gaillard, J.L., Berche, P., Mounier, J., Richard, S. and Sansonetti, P. (1987). In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect Immun.* **55,** 2822–2829.

Gaillard, J.-L., Berche, P. and Sansonetti, P. (1986). Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes. Infect. Immun*. **52,** 50–55.

Gedde, M.M., Higgins, D.E., Tilney, L.G. and Portnoy D.A. (2000). Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes. Infect. Immun*. **68,** 999–1003.

Geoffroy, C., Gaillard, J.-L., Alouf, J.E. and Berche, P. (1987). Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes. Infect. Immun*. **55,** 1641–1646.

Geoffroy, C., Gaillard, J.-L., Alouf, J.E. and Berche, P. (1989). Production of thiol-dependent hemolysins by *Listeria monocytogenes* and related species. *J. Gen. Microbiol*. **135,** 481–487.

Gekara, N.O. and Weiss, S. (2004). Lipid rafts clustering and signaling by listeriolysin O. *Biochem. Soc. Trans*. **32,** 712–714.

Gholizadeh, Y., Juvin, M., Beretti, J.-L., Berche, P. and Gaillard, J.-L. (1997). Culture-negative listeriosis of the central nervous system diagnosed by detection of antibodies to listeriolysin O. *Eur. J. Clin. Microbiol. Infect. Dis*. **16,** 176–178.

Gholizadeh, Y., Poyart, C., Juvin, M., Beretti, J.L., Croizé, J. Berche, P. and Gaillard, J.L. (1996). Serodiagnosis of listeriosis based upon detection of antibodies against recombinant truncated forms of listeriolysin O. *J. Clin. Microbiol*. **34,** 1391–1395.

Giddings, K.S., Johnson, A.E. and Tweten, R.K. (2003). Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins. *P. Natl. Acad. Sci. USA* **100,** 11315–11320.

Giddings, K.S., Zhao, J., Sims, P.J. and Tweten, R.K. (2004). Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin. *Nat. Struct. Mol. Biol*. **11,** 1173–1178.

Gilbert, R.J., Rossjohn, J., Parker, M.W., Tweten, R.K., Morgan, P.J., Mitchell, T.J., Errington, N., Rowe, A.J., Andrew, P.W. and Byron, O. (1998). Self-interaction of pneumolysin, the pore-forming protein toxin of *Streptococcus pneumoniae. J. Mol. Biol*. **284,** 1223–1237.

Gilbert, R.J.C., Jiménez, J.L., Chen, S., Tickel, I.J., Rossjohn, J., Parker, M., Andrew, P.W. and Saibil, H.R. (1999). Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae. Cell*, **97,** 647–655.

Girard, K.F., Sbarra, A.J. and Bardawil, W.A. (1963). Serology of *Listeria monocytogenes*. I. Characteristics of the soluble hemolysin. *J. Bacteriol*. **85,** 349–355.

Glomski, I.J., Decatur, A.L. and Portnoy, D.A. (2003). *Listeria monocytogenes* mutants that fail to compartmentalize listerolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses. *Infect. Immun*. **71,** 6754–6765.

Glomski, I.J., Gedde, M.M., Tsang, A.W., Swanson, J.A. and Portnoy, D.A. (2002). The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. *J. Cell. Biol*. **156,** 1029–1038.

Grenningloh, R., Darji, A., Wehland, J., Chakraborty, T. and Weiss, S. (1998). Listeriolysin and IrpA are major protein targets of the human humoral reponse against *Listeria monocytogenes. Infect. Immun*. **65,** 3976–3980.

Grillot-Courvalin, C., Goussard, S., Huetz, F., Ojcius, D.M. and Courvalin, P. (1998). Functional gene transfer from intracellular bacteria to mammalian cells. *Nat. Biotechnol*. **16,** 862–866.

Gunn, G.R., Zubair, A., Peters, C., Pan, Z.K., Wu, T.C. and Paterson, Y. (2001). Two *Listeria monocytogenes* vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16. *J. Immunol*. **167,** 6471–6479.

Guzman, C.A., Domann, E., Rhode, M., Bruder, D., Darji, A., Weiss, S., Wehland, J., Chakraborty, T. and Timmis, K. N. (1996). Apoptosis of mouse dendritic cells is triggered by listeriolysin, the major virulence determinant of *Listeria monocytogenes. Mol. Microbiol*. **20,** 119–126.

Guzman, C.A., Saverino, D., Medina, E., Fenoglio, D., Gerstel, B., Merlo, A., Li Pira, G., Buffa, F., Chakraborty, T. and Manca, F. (1998). Attenuated *Listeria monocytogenes* carrier strains can deliver an HIV-1 gp120 T helper epitope to MHC class II-restricted human CD4+ T cells. *Eur. J. Immunol*. **28,** 1807–1814.

Guzman, C.A., Weiss, S. and Chakraborty, T. (1997). *Listeria monocytogenes*—a promising vaccine carrier to evoke cellular immune responses. In: Gram-positive bacteria as vaccine vehicles for mucosal immunization (eds. G. Pozzi and J. Wells) 145–173. Landes Biosciences, Austin.

Haas, A., Dumbsky, M. and Kreft. J. (1992). Listeriolysin genes: complete sequence of *ilo* from *Listeria ivanovii* and of *lso* from *Listeria seeligeri. Biochim. Biophys. Acta* **1130,** 81–84.

Harty, J.T. and Bevan, M.J. (1992). CD8+ T cells specific for a single nonamer epitope of *Listeria monocytogenes* are protective *in vivo. J. Exp. Med*. **175,** 1531–1540.

Harvey, P.C. and Faber, J.E. (1941). Some biochemical reactions of the *Listerella* group. *J. Bacteriol*. **41,** 45–46.

Heuck, A.P., Hotze, E.M., Tweten, R.K. and Johnson, A.E. (2000). Mechanism of membrane insertion of a multimeric beta-barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. *Mol. Cell*. **6,** 1233–1242.

Heuck, A.P., Tweten, R. and Johnson, A.E. (2003). Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J. Biol. Chem*. **278,** 31218–31255.

Hess, J., Gentschev, I., Miko, D., Welzel, M., Ladel, C., Goebel, W. and Kaufmann, S.H. (1996). Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine-induced protection against listeriosis. *Proc. Natl. Acad. Sci. USA* **93,** 1458–1463.

Hess, J., Grode, L., Gentschev, I., Fensterle, J,. Dietrich, G., Goebel, W. and Kaufmann, S.H. (2000). Secretion of different listeriolysin cognates by recombinant attenuated *Salmonella typhimurium*: superior efficacy of hemolytic over non-hemolytic constructs after oral vaccination. *Microbes Infect*. **2,** 1799–1806.

Hess, J., Miko, D., Catic, A., Lehmensiek, V., Russell, D.G. and Kaufmann, S.H. (1998). *Mycobacterium bovis* Bacille Calmette-Guerin strains secreting listeriolysin of *Listeria monocytogenes. Proc. Natl. Acad. Sci. USA*, **95,** 5299–5304.

Hiltbold, E.M., Safley, S.A. and Ziegler, H.K. (1996). The presentation of class I and class II epitopes of listeriolysin O is regulated by intracellular localization and by intercellular spread of *Listeria monocytogenes. J. Immunol*. **157,** 1163–1175.

Hotze, E.M., Heuck, A.P., Czaikowsky, D.M., Shao, Z., Johnson, A.E. and Tweten, R.K. (2002). Monomer-monomer interactions drive the prepore to pore conversion of a β-barrel-forming, cholesterol-dependent cytolysin. *J. Biol. Chem*. **277,** 11597–11605.

Hu, P.Q., Tuma-Warrino, R.J., Bryan, M.A., Mitchell, K.G., Higgins, D.E., Watkins, S.C. and Salter, R.D. (2004). *Escherichia coli* expressing recombinant antigen and listeriolysin O stimulate class I-restricted CD8+ T cells following uptake by human APC. *J. Immunol*. **172,** 1595–1601.

Ikonomidis, G., Frankel, F.R., Portnoy D.A. and Paterson, Y. (1995). *Listeria monocytogenes*: A novel live vaccine vector. In: *Vaccines 95* (eds. R.M. Chanock, F. Brown, H.S. Ginsberg and E. Norrby), pp. 317–326. Harbor Laboratory Press, Cold Spring.

Ikonomidis, G., Paterson, Y., Kos, F.J. and Portnoy, D.A. (1994). Delivery of a viral antigen to the class I processing and presentation pathway by *Listeria monocytogenes. J. Exp. Med*. **180,** 2209–2218.

Ikonomidis, G., Portnoy, D.A., Gerhard, W. and Paterson, Y. (1997). Influenza-specific immunity induced by recombinant *Listeria monocytogenes* vaccines. *Vaccine* **15,** 433–440.

Ito, Y., Kawamura, I., Kohda, C., Baba, H., Kimoto, T., Watanabe, I., Nomura, T. and Mitsuyama, M. (2001). Difference in cholesterol-binding and cytolytic activities between listeriolysin O and seeligeriolysin O: a possible role of alanine residue in tryptophan-rich undecapeptide. *FEMS Microbiol. Lett*. **203,** 185–189.

Ito, Y., Kawamura, I., Kohda, C., Baba, H., Nomura, T., Kimoto, T., Watanabe, I. and Mitsuyama, M. (2003). Seeligeriolysin O, a cholesterol-dependent cytolysin of *Listeria seeligeri*, induces gamma interferon from spleen cells of mice. *Infect. Immun*. **71,** 234–241.

Jacobs, T., Cima-Cabal, M.D., Darji, A., Mendez, F.J., Vazquez, F., Jacobs, A.A., Shimada, Y., Ohno-Iwashita, Y., Weiss, S. and de los Toyos, J.R. (1999). The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding. *FEBS Lett*. **459,** 463–466.

Jacobs, T., Darji, A., Frahm, N., Rohde, M., Wehland, J., Chakraborty, T. and Weiss, S. (1998). Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol. Microbiol*. **28,** 1081–1089.

Jenkins, E.M., Njoku-Obi, A.N. and Adams, E.W. (1964). Purification of the soluble hemolysin of *Listeria monocytogenes. J. Bacteriol*. **88,** 418–424.

Jenkins, E.M. and Watson, B.B. (1971). Extracellular antigens from *Listeria monocytogenes*. I. Purification and resolution of hemolytic and lipolytic antigens from culture filtrates of *Listeria monocytogenes. Infect. Immun*. **3,** 589–594.

Jensen, E.R., Selvakumar, R., Shen, H., Ahmed, R., Wettstein, F.O. and Miller, J.F. (1997). Recombinant *Listeria monocytogenes* vaccination eliminates papillomavirus-induced tumors and prevents papilloma formation from viral DNA. *J. Virol*. **71,** 8467–8674.

Jones, S. and Portnoy, D.A. (1994). Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. *Infect Immun*. **62,** 5608–5613.

Jones, S., Preiter, K. and Portnoy, D.A. (1996). Conversion of an extracellular cytolysin into a phagosome-specific lysin, which supports the growth of an intracellular pathogen. *Mol. Microbiol*. **21,** 1219–1225.

Karunasagar, I., Lampidis, R., Goebel, W. and Kreft, J. (1997). Complementation of *Listeria seeligeri* with the plcA-prfA genes from *L. monocytogenes* activates transcription of seeligerolysin and leads to bacterial escape from the phagosome of infected mammalian cells. *FEMS Microbiol. Lett*. **146,** 303–310.

Kayal, S., Lilienbaum, A., Join-Lambert, O., Li, X., Israel, A. and Berche, P. (2002). Listeriolysin O secreted by *Listeria monocytogenes* induces NF-kappaB signaling by activating the IkappaB kinase complex. *Mol. Microbiol*. **44,** 1407–1419.

Kayal, S., Lilienbaum, A., Poyart, C., Memet, S., Israel, A. and Berche, P. (1999). Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kB and up-regulation of adhesion molecules and chemokines. *Mol. Microbiol*. **31,** 1709–1722.

Kathariou, S., Metz, P., Hof, H. and Goebel, W. (1987). Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes. J. Bacteriol*. **169,** 1291–1297.

Kaufmann, S.H.E. (1993). Immunity to intracellular bacteria. *Annu. Rev. Immunol*. **11,** 129–163.

Kingdon, G.C. and Sword, C.P. (1970). Effects of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. *Infect. Immun*. **1,** 356–362.

Kohda, C., Kawamura, I., Baba, H., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I. and Mitsuyama, M. (2002). Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect. Immun*. **70,** 1334–1341.

Kreft, J. and Vázquez-Boland, J.A. (2001). Regulation of virulence genes in *Listeria. Int. J. Med. Microbiol*. **291,** 145–157.

Krüll, M., Nost, R., Hippenstiel, S., Domann, E., Chakraborty, T. and Suttorp, N. (1997). *Listeria monocytogenes* potently induces up-regulation of endothelial adhesion molecules and neutrophil adhesion to cultured human endothelial cells. *J. Immunol*. **159,** 1970–1976.

Kuhn, M. and Goebel, W. (1994). Induction of cytokines in phagocytic mammalian cells infected with virulent and avirulent *Listeria* strains. *Infect. Immun*. **62,** 348–356.

Kuhn, M., Kathariou, S. and Goebel, W. (1988). Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes. Infect. Immun*. **56,** 79–82.

Lee, K.D., Oh, Y.K., Portnoy, D.A. and Swanson, J.A. (1996). Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes*. *J. Biol. Chem*. **271,** 7249–7252.

Leimeister-Wächter, M. and Chakraborty, T. (1989). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes, Listeria ivanovii*, and *Listeria seeligeri*. *Infect. Immun*. **57,** 2350–2357.

Lety, M.A., Frehel, C., Beretti, J.L., Berche, P. and Charbit, A. (2003). Modification of the signal sequence cleavage site of listeriolysin O does not affect protein secretion but impairs the virulence of *Listeria monocytogenes*. *Microbiology* **149,** 1249–1255.

L'hopital, S., Marly, J. Pardon, P. and Berche, P. (1993). Kinetics of antibody production against listeriolysin O in sheep with listeriosis. *J. Clin. Microbiol*. **31,** 1537–1540.

Lieberman, J. and Frankel, F.R. (2002). Engineered *Listeria monocytogenes* as an AIDS vaccine. *Vaccine* **20,** 2007–2010.

Low, J.C. and Donachie, W. (1991). Clinical and serum antibody responses in lambs to infection by *Listeria monocytogenes*. *Res. Vet. Sci*. **51,** 185–192.

Low, J.C., Davies, R.C. and Donachie, W. (1992). Purification of listeriolysin O and development of an immunoassay for diagnosis of listeric infections in sheep. *J. Clin. Microbiol*. **30,** 2705–2708.

Madden, J.C., Ruiz, N., Caparon, M. (2001). Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152.

Mandal, M. and Lee, K.D. (2002). Listeriolysin O liposome-mediated cytosolic delivery of macromolecule antigen *in vivo*: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection. *Biochim. Biophys. Acta*. **1563,** 7–17.

Mandal, M., Mathew, E., Provoda, C. and Lee, K.-D. (2003) Delivery of macromolecules into cytosol using liposomes containing hemolysin. *Methods Enzymol*. **372,** 319–339.

Marco, A.J., Domingo, M., Prats, M., Briones, V., Pumarola, M. and Domínguez, L. (1991) Pathogenesis of lymphoid lesions in murine experimental listeriosis. *J. Comp. Path*. **105,** 1–15.

Mata, M. and Paterson, Y.J. (1999). Th1 T cell responses to HIV-1 Gag protein delivered by a *Listeria monocytogenes* vaccine are similar to those induced by endogenous listerial antigens. *Immunol*. **163,** 1449–1456.

Mata, M., Yao, Z.J., Zubair, A., Syres, K. and Paterson, Y. (2001). Evaluation of a recombinant *Listeria monocytogenes* expressing an HIV protein that protects mice against viral challenge. *Vaccine* **19,** 1435–1445.

Mathew, E., Hardee, G.E., Bennett, C.F. and Lee, K.D. (2003). Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes. *Gene Ther*. **10,** 1105–1115.

McCaffrey, R.L., Fawcett, P., O'Riordan, M., Lee, K.D., Havell, E.A., Brown, P.O. and Portnoy, D.A. (2004). A specific gene expression program triggered by Gram-positive bacteria in the cytosol. *Proc. Natl. Acad. Sci. USA* **101,** 11386–11391.

Merrick, J.C., Edelson, B.T., Bhardwaj, V., Swanson, P.E. and Unanue, E.R. (1997). Lymphocyte apoptosis during early phase of *Listeria* infection in mice. *Am. J. Pathol*. **151,** 785–792.

Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J.L. and Cossart, P. (1987). Identification of the structural gene encoding the SH-activated hemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. *Infect. Immun*. **56,** 766–772.

Mengaud, J., Vicente, M.-F., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.-C. and Cossart, P. (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. *Infect. Immun*. **56,** 766–772.

Michel, E., Reich, K.A., Favier, R., Berche, P. and Cossart, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino acid substitutions in listeriolysin O. *Mol. Microbiol*. **4,** 3609–3619.

Miettinen, A. and Husu, J. (1991). Antibodies to listeriolysin O reflect the acquired resistance to *Listeria monocytogenes* in experimentally infected goats. *FEMS Microbiol. Lett*. **61,** 181–186.

Morgan, P.J., Hyman, S.C., Byron, O., Andrew, P.W., Mitchell, T.J. and Rowe, A.J. (1994). Modeling the bacterial protein toxin, pneumolysin, in its monomeric and oligomeric form. *J. Biol. Chem*. **269,** 25315–25320.

Nakamura, M., Sekino-Suzuki, N., Mitsui, K. and Ohno-Iwashita, Y. (1998). Contribution of tryptophan residues to the structural changes in perfringolysin O during interaction with liposomal membranes. *J. Biochem*. **123,** 1145–1155.

Nato, F., Reich, K., Lhopital, S. Rouyre, S., Geoffroy, C., Mazie, J.C. and Cossart. P. (1991). Production and characterization of neutralizing and non-neutralizing monoclonal antibodies against listeriolysin O. *Infect. Immun*. **59,** 4641–4646.

Nishibori, T., Xiong, H., Kawamura, I. Arakaea, M. and Mitsuyama, M. (1996). Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells. *Infect. Immun*. **64,** 3188–3195.

Njoku-Obi, A.N., Jenkins, E.M., Njoku-Obi, J.C., Adams, J. and Covington, V. (1963). Production and nature of *Listeria monocytogenes* hemolysins. *J. Bacteriol*. **86,** 1–8.

Nogva, H.K, Rudi, K., Naterstad, K., Holck, A. and Lillehaug, D. (2000). Application of 5'-nuclease PCR for quantitative detection of *Listeria monocytogenes* in pure cultures, water, skim milk, and unpasteurized whole milk. *Appl. Environ. Microbiol*. **66,** 4266–4271.

Nomura, T., Kawamura, I., Tsuchiya, K., Kohda, C., Baba, H., Ito, Y., Kimoto, T., Watanabe, I. and Mitsuyama, M. (2002). Essential role of interleukin-12 (IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. *Infect. Immun*. **70,** 1049–1055.

O'Riordan, M., Yi, C.H., Gonzales, R., Lee, K.D. and Portnoy, D.A. (2002). Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. *Proc. Natl. Acad. Sci. USA*. **99,** 13861–1386.

Paglia, P., Arioli, I., Frahm, N., Chakraborty, T., Colombo, M.P. and Guzman, C.A. (1997). The defined attenuated *Listeria monocytogenes* delta *mp12* mutant is an effective oral vaccine carrier to trigger a long-lasting immune response against a mouse fibrosarcoma. *Eur. J. Immunol*. **27,** 1570–1575.

Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J. and Bhakdi, S. (1998a). Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. *EMBO J*. **6,** 1598–1605.

Palmer, M., Vulicevic, I., Saweljew, P., Valeva, P., Kehoe, M. and Bhakdi, S. (1998b). Streptolysin O: A proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* **37,** 2378–2383.

Pamer, E.G., Harty, J.T. and Bevan, M. (1991). Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. *Nature* **353,** 852–855.

Pan, Z.K., Ikonomidis, G., Pardoll, D. and Paterson, Y. (1995). Regression of established tumors in mice mediated by the oral administration of a recombinant *Listeria monocytogenes* vaccine. *Cancer Res*. **55,** 4776–4779.

Peters, C., Peng, X., Douven, D., Pan, Z.K. and Paterson, Y. (2003). The induction of HIV Gag-specific CD8+ T cells in the spleen and gut-associated lymphoid tissue by parenteral or mucosal immunization with recombinant *Listeria monocytogenes* HIV Gag. *J. Immunol*. **170,** 5176–5187.

Pinkney, M., Beachey, E. and Kehoe, M. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun*. **57,** 2553–2558.

Polekina, G., Giddings, K.S., Tweten, R.K. and Parker, M.W. (2005). Insights into the action of the superfamily of cholesterol-dependent cytoslysins from studies of intermedilysin. *P. Natl. Acad. Sci. USA* **102,** 600–605.

Portnoy, D.A., Auerbuch, V. and Glomski, I.J. (2002). The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *J. Cell. Biol*. **158,** 409–414.

Portnoy, D.A., Jacks, P.S. and Hinrichs, D.J. (1988). Role of hemolysin for the intracellular growth of *Listeria monocytogenes. J. Exp. Med*. **167,** 1459–1471.

Portnoy, D.A., Tweten, R.K., Kehoe, M. and Bielecki, J. (1992). Capacity of listeriolysin O, streptolysin O, and prefringolysin O to mediate growth of *Bacillus subtilis* within mammalian cells. *Infect. Immun*. **60,** 2710–2717.

Provoda, C.J., Stier, E.M. and Lee, K.D. (2003). Tumor cell killing enabled by listeriolysin O liposome-mediated delivery of the protein toxin gelonin. *J. Biol. Chem*. **278,** 35102–35108.

Radford, K.J., Jackson, A.M., Wang, J.H., Vassaux, G. and Lemoine, N.R. (2003). Recombinant *E. coli* efficiently delivers antigen and maturation signals to human dendritic cells: presentation of MART1 to CD8+ T cells. *Int. J. Cancer* **105,** 811–819.

Ramachandran, R., Heuck, A.P., Tweten, R.K. and Johnson, A.E. (2002). Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin. *Nat. Struct. Biol*. **9,** 823–827.

Ramachandran, R., Tweten, R.K. and Johnson, A.E. (2004). Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit β-strand alignment. *Nat. Struct. Biol*. **11,** 697–705.

Repp, H., Pamukci, Z., Koschinski, A., Domann, E., Darji, A., Birringer, J., Brockmeier, D., Chakraborty, T. and Dreyer, F. (2002). Listeriolysin of *Listeria monocytogenes* forms Ca2+-permeable pores leading to intracellular Ca2+ oscillations. *Cell. Microbiol*. **4,** 483–491.

Rodriguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J.A. and Pla, M. (2004). Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly, iap*, and *lin02483* targets and AmpliFluor technology. *Appl. Environ. Microbiol*. **70,** 1366–1377.

Rose, F., Zeller, S.A., Chakraborty, T., Domann, E., Machleidt, T., Kronke, M., Seeger, W., Grimminger, F. and Sibelius, U. (2001). Human endothelial cell activation and mediator release in response to *Listeria monocytogenes* virulence factors. *Infect. Immun*. **69,** 897–905.

Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K. and Parker, M.W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89,** 685–692.

Russmann, H., Gerdemann, U., Igwe, E.I., Panthel, K., Heesemann, J., Garbom, S., Wolf-Watz, H. and Geginat, G. (2003). Attenuated *Yersinia pseudotuberculosis* carrier vaccine for simultaneous antigen-specific CD4 and CD8 T cell induction. *Infect. Immun*. **71,** 3463–3472.

Russmann, H., Igwe, E.I., Sauer, J., Hardt, W.D., Bubert, A. and Geginat, G. (2001). Protection against murine listeriosis by oral vaccination with recombinant *Salmonella* expressing hybrid *Yersinia* type III proteins. *J. Immunol*. **167,** 357–65.

Safley, S.A., Cluff, C.W., Marshall, N.E. and H.K. Ziegler. (1991). Role of listeriolysin O in the T lymphocyte response to infection with *Listeria monocytogenes. J. Immunol*. **146,** 3604–3616.

San Mateo, L.R., Chua, M.M., Weiss, S.R. and Shen, H. (2002). Perforin-mediated CTL cytolysis counteracts direct cell-cell spread of *Listeria monocytogenes. J. Immunol*. **169,** 5202–5208.

Saunders, F.K., Mitchell, T.J. Walker, J.A. Andrew, P.W. and Boulnois, G.J. (1989). Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect. Immun*. **57,** 2547–2552.

Schmid M.W., Ng E.Y.W., Lampidis, R., Emmerth, M., Walcher, M., Kreft, J., Goebel, W., Wagner, M. and Schleifer, K.H. (2005) Evolutionary history of the genus *Listeria* and its virulence genes. *Syst. Appl. Microbiol*. **28,** 1–18.

Schüller, S., Kügler, S. and Goebel, W. (1998). Suppression of major histocompatibility complex class I and class II gene expression in *Listeria monocytogenes*-infected murine macrophages. *FEMS Immunol. Med. Microbiol*. **20,** 289–299.

Sekino-Suzuki, N., Nakamura, M., Mitsui, K.I. and Ohno-Iwashita, Y. (1996). Contribution of individual triptophane residues to the structure and activity of theta-toxin (perfringolysin O), a cholesterol-binding cytolysin. *Eur. J. Biochem*. **241,** 941–947.

Sekiya, K., Satoh, R. Danbara, H. and Futaesaku, Y. (1993). A ring-shaped structure with a crown formed by streptolysin O on the erythrocyte membrane. *J. Bacteriol*. **175,** 5953–5596.

Shatursky, O, Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E. and Tweten, R.K. (1999). The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99,** 293–299.

Shen, H., Slifka, M.K., Matloubian, M., Jensen, E.R., Ahmed, R. and Miller, J.F. (1995). Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral, cell-mediated immunity. *Proc. Natl. Acad. Sci. USA* **92,** 3987–3991.

Shen, H., Tato, C.M. and Fan, X. (1998). *Listeria monocytogenes* as a probe to study cell-mediated immunity. *Curr. Opin. Immunol*. **10,** 450–458.

Shepard, L.A., Heuck, A., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., Johnson, A.E. and Tweten, R.K. (1998). Identification of a membrane-spanning domain of the thiol-activated, pore-forming toxin *Clostridium perfringens* perfringolysin O: an α-helical to β-sheet transition identified by fluorescence spectroscopy. *Biochemistry* **37,** 14563–14574.

Shepard, L.A., Shatursky, O., Johnson, A.E. and Tweten, R.K. (2000). The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane beta-hairpins. *Biochemistry* **39,** 10284–10293.

Shimada, Y., Maruya, M., Iwashita, S. and Ohno-Iwashita, Y. (2002). The C-terminal domain of perfringolysin O is an essential cholesterol-binding unit targeting to cholesterol-rich microdomains. *Eur. J. Biochem*. **269,** 6195–6203.

Shimada, Y., Nakamura, M., Naito, Y., Nomura, K. and Ohno-Iwashita, Y. (1999). C-terminal amino acid residues are required for the folding and cholesterol binding property of perfringolysin O, a pore-forming cytolysin. *J. Biol. Chem*. **274,** 18536–18542.

Sibelius, U., Chakraborty, T., Krogel, B., Wolf, J., Rose, F., Schmidt, R., Wehland, J., Seeger, W. and Grimminger, F. (1996a). The listerial exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C synergize to elicit endothelial cell phosphoinositide metabolism. *J. Immunol*. **157,** 4055–4060.

Sibelius, U., Rose, F., Chakraborty, T., Darji, A., Wehland, J., Weiss, S., Seeger, W. and Grimminger, F. (1996b). Listeriolysin is a potent inducer of the phosphatidylinositol response and lipid mediator generation in human endothelial cells. *Infect. Immun*. **64,** 674–676.

Sibelius, U., Schulz, E.C., Rose, F., Hattar, K., Jacobs, T., Weiss, S., Chakraborty, T., Seeger, W. and Grimminger, F. (1999). Role of *Listeria monocytogenes* exotoxins listeriolysin and phosphatidylinositol-specific phospholipase C in activation of human neutrophils. *Infect. Immun*. **67,** 1125–1130.

Siddique, I.H., I-Fong Lin, M.S. and Chung, R.A. (1974). Purification and characterization of hemolysin produced by *Listeria monocytogenes*. *Am. J. Vet. Res*. **35,** 289–296.

Sirard, J.-C., Fayolle, C., de Chastellier, C., Mock, M., Leclerc, C. and Berche, P. (1997). Intracytoplasmic delivery of listeriolysin O by a vaccinal strain of *Bacillus anthracis* induces CD8-mediated protection against *Listeria monocytogenes*. *J. Immunol*. **159,** 4435–4443.

Smyth, J. and Duncan, J.L. (1978). Thiol-activated (oxygen labile) cytolysins. In: *Bacterial Toxins and Cell Membranes* (eds. J. Jeljaszewicz and T. Wadström), pp. 129–183. Academic Press Ltd, London.

Spreng, S., Dietrich, G., Niewiesk, S., ter Meulen, V., Gentschev, I. and Goebel, W. (2000). Novel bacterial systems for the delivery of recombinant protein or DNA. *FEMS Immunol. Med. Microbiol*. **27,** 299–304.

Stockinger, S., Materna, T., Stoiber, D., Bayr, L., Steinborn, R., Kolbe, T., Unger, H., Chakraborty, T., Levy, D.E., Muller, M. and Decker, T. (2002). Production of type I IFN sensitizes macrophages to cell death induced by *Listeria monocytogenes*. *J. Immunol*. **169,** 6522–6529.

Tanabe, Y., Xiong, H., Nomura, T., Arakawa, M. and Mitsuyama, M. (1999). Induction of protective T cells against *Listeria monocytogenes* in mice by immunization with a listeriolysin O-negative avirulent strain of bacteria and liposome-encapsulated listeriolysin O. *Infect Immun*. **67,** 568–575.

Tang, P., Rosenshine, I., Cossart, P. and Finlay, B.B. (1996). Listeriolysin O activates mitogen-activated protein kinase in eucaryotic cells. *Infect. Immun*. **64,** 2359–2361.

Tang, P., Rosenshine, I. and Finlay, B.B. (1994). *Listeria monocytogenes*, an invasive bacterium, stimulates MAP kinase upon attachment to epithelial cells. *Mol. Biol. Cell*. **5,** 455–464.

Thompson, R.J., Bouwer, H.G., Portnoy, D.A. and Frankel, F.R. (1998). Pathogenicity and immunogenicity of a *Listeria monocytogenes* strain that requires D-alanine for growth. *Infect. Immun*. **66,** 3552–3561.

Unanue, E.R. (1997). Studies in listeriosis show the strong symbiosis between the innate cellular system and the T cell response. *Immunol Rev*. **158,** 11–25.

Vázquez-Boland, J.-A., Dominguez, L., Rodriguez-Ferri, E.F. and Suarez, G. (1989a). Purification and characterization of two *Listeria ivanovii* cytolysins, a sphingomyelinas C and a thiol-activated toxin (ivanolysin O). *Infect. Immun*. **57,** 3928–3935.

Vázquez-Boland, J.-A., Dominguez, L., Rodriguez-Ferri, E.F., Fernandez-Garayzabal, J.F. and Suarez, G. (1989b). Preliminary evidence that different domains are involved in cytolytic activity and receptor (cholesterol) binding in listeriolysin O, the *Listeria monocytogenes* thiol-activated toxin. *FEMS Microbiol. Lett*. **65,** 95–100.

Vázquez-Boland, J.A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J. and Goebel, W. (2001a). Pathogenicity islands and virulence evolution in *Listeria*. *Microbes Infect*. **3,** 571–584.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J. and Kreft, J. (2001b). *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev*. **14,** 584–640.

Villanueva, M.S., Sijts, A.J.A.M. and Pamer, E. (1995). Listeriolysin is processed efficiently into an MHC class I-associated epitope in *Listeria monocytogenes*-infected cells. *J. Immunol*. **155,** 5227–5233.

Wadsworth, S.J. and Goldfine, H. (2002). Mobilization of protein kinase C in macrophages induced by *Listeria monocytogenes* affects its internalization and escape from the phagosome. *Infect Immun*. **70,** 4650–4660.

Waheed, A.A., Shimada, Y., Heijnen, H., Nakamura, M., Inomata, M., Hayashi, M., Iwashita, S., Slot, J.W. and Ohno-Iwashita, Y. (2001). Selective binding of perfringolysin to cholesterol-rich membrane microdomains (rafts). *P. Natl. Acad. Sci. USA* **98,** 4926–4931.

Walev, I., Bhakdi, S.C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin O. *Proc. Natl. Acad. Sci. USA* **98,** 3185–3190.

Watson, B.B. and Lavizzo, J.C. (1973). Extracellular antigens from *Listeria monocytogenes*. II. cytotoxicity of hemolytic and lipolytic antigens of *Listeria* for cultured mouse macrophages. *Infect. Immun*. **7,** 753–758.

Weiglein, I., Goebel, W., Troppmair, J., Rapp, U.R., Demuth, A. and Kuhn, M. (1997). *Listeria monocytogenes* infection of HeLa cells results in listeriolysin O-mediated transient activation of the Raf-MEK-MAP kinase pathway. *FEMS Microbiol. Lett*. **148,** 189–195.

Weiskirch, L.M. and Paterson, Y. (1997). *Listeria monocytogenes*: a potent vaccine vector for neoplastic and infectious disease. *Immunol. Rev*. **158,** 159–169.

Yoshida, A., Nagata, T., Uchijima, M. and Koide, Y. (2001). Protective CTL response is induced in the absence of CD4+ T cells and IFN-gamma by gene gun DNA vaccination with a minigene encoding a CTL epitope of *Listeria monocytogenes*. *Vaccine* **19,** 4297–4306.

Yoshikawa, H., Kawamura, I., Fujita, M., Tsukada, H., Arawaka, M. and Mitsuyama, M. (1993). Membrane damage and interleukin-1 production in murine macrophages exposed to listeriolysin O. *Infect. Immun*. **61,** 1334–1339.

Zenewicz, L.A., Skinner, J.A., Goldfine, H. and Shen, H. (2004). *Listeria monocytogenes* virulence proteins induce surface expression of Fas ligand on T lymphocytes. *Mol. Microbiol*. **51,** 1483–1492.

CHAPTER

# 41

# *Enterococcus faecalis* cytolysin toxin

*Karen Carniol and Michael S. Gilmore*

The cytolysin toxin, produced by the Gram-positive bacterium *Enterococcus faecalis*, is unique among bacterial toxins in its ability to lyse both bacterial cells and eukaryotic cells. It was first identified as a hemolysin, but further studies classified it as a divergent member of the lantibiotic family of bacteriocins. This chapter describes what is known about the range of target cells, the molecular determinants of cytolysin activity, the mobility of the cytolysin-encoding locus, regulation of the expression of cytolysin activity, and the role of cytolysin in enterococcal virulence.

## BACKGROUND ON ENTEROCOCCI

Enterococci exist largely as commensal organisms in the gastrointestinal tracts of a wide variety of animals. They thrive in this environment, achieving an average density of roughly $10^6$ colony-forming units per gram of feces (Noble, 1978). Enterococci can also be pathogens colonizing blood, vascular tissue, and retinal tissue (Gilmore *et al*., 2002). Two species of Enterococcus—*faecalis* and *faecium*—are found frequently as the causative agents of nosocomial infections and have emerged as the leading cause of surgical site infections, the second-leading cause of bloodstream infections, the third-leading cause of nosocomial urinary tract infections, and are frequent isolates from subacute endocarditis (Richards *et al*., 2000). Enterococcal infections are of particular concern since strains that are resistant to virtually all available antibiotic therapies have emerged over the last two decades (Huycke *et al*., 1998).

Antibiotic resistance traits are most widespread in strains of *E. faecium*, yet *E. faecalis* strains comprise 70–80% of all nosocomial enterococcal infections (Jett *et al*., 1994; Jones *et al*., 2004). The pathogenicity of *E. faecalis* strains may well be attributable to a larger repertoire of non-antibiotic resistance virulence factors, including the cytolysin toxin (Elsner *et al*., 2000).

## PRE–MOLECULAR–ERA STUDIES OF CYTOLYSIN

E.W. Todd's 1934 study of the hemolytic activity of group-D streptococcal strains (subsequently called *Streptococcus zymogenes*, now called *Entercoccus faecalis*) is generally recognized as the first characterization of what is now known to be the Enterococcus cytolysin. Todd observed hemolysis on blood agar, but was not able to recover the hemolytic activity from liquid culture supernatants, a paradox that inspired the designation "pseudohemolysin." The reasons for this apparent paradox are now better understood and are described below. Subsequent development of a horseflesh infusion medium that could support recovery of active toxin from culture filtrates enabled Todd to characterize the toxin as acid and heat labile, oxygen stable, and non-immunogenic in rabbits (Todd, 1934). Studies by Kobayashi (1940), Roelofsen *et al*. (1964), and Basinger and Jackson (1968) defined a spectrum of susceptibility to cytolysin among erythrocytes from different species. Human, horse, dog, rabbit, and mouse erythrocytes are the most susceptible to lysis, sheep erythrocytes are largely resistant, and goose erythrocytes are completely resistant.

In 1949 Sherwood *et al*. noted that a majority of hemolytic enterococcal strains also produced a bacteriocin active against other streptococcal strains.

Succeeding studies (Stark, 1960; Brock *et al*., 1963; Jett and Gilmore, 1990) demonstrated that several Gram-positive bacteria are susceptible to this bacteriocin activity, including *Staphylococcus, Streptococcus, Clostridium, Leuconostoc, Lactobacillus, Mirococcus, Corynebacterium*, and some *Bacillus* species. *Bacillus subtilis* is only partially susceptible and *Bacillus polymyxa* is completely resistant, as are Gram-negative species, such as *Escherichia coli* and *Proteus vulgaris*. The properties of cells susceptible to cytolysin versus those that are refractory, and the basis for intermediate degrees of sensitivity, are not clear.

Brock *et al*. (1963) noted the complete correspondence between hemolytic activity and the bacteriocin activity elaborated by *S. zymogenes* (hemolytic *E. faecalis*) and speculated that the activities were due to the same molecular entity. Brock and Davie (1963) furnished proof for this speculation by demonstrating that UV irradiation of this strain caused simultaneous loss of hemolytic and bacteriocin activity, and that a reversion event restoring one activity was always concomitant with restoration of the other activity. The term *cytolysin* was adopted to reflect the dual hemolytic/bacteriocin activity of this particular toxin (Gilmore, 1991).

Granato and Jackson (1969) demonstrated that cytolytic activity required more than one factor. Nonhemolytic mutants (obtained by NTG mutagenesis) were assayed for the ability to complement each other extracellularly in pairwise cross-streaks on blood agar. The mutants fell into two complementation groups with distinctive properties—one group appeared defective in a lytic component, the other defective in an activating component. Refined genetic techniques and nucleotide sequence analysis of the cytolysin locus would subsequently elucidate the molecular basis for this astute observation.

## THE CYTOLYSIN LOCUS

The cytolysin locus was initially observed to be encoded on conjugative plasmids in *E. faecalis* strains from infections, as first demonstrated by plasmid curing and broth mating experiments (Dunny and Clewell, 1975; Jacob *et al*., 1975; Ike and Clewell, 1992). At least eight different conjugative plasmids, falling into at least three incompatibility groups, have been observed to encode the cytolysin determinants (Ike and Clewell, 1992). These plasmids may be transferred between strains via a pheromone-responsive conjugation pathway (Clewell *et al*., 1982; Huycke *et al*., 1992; Clewell, 1993; Dunny and Leonard, 1997). Transposon mutagenesis mapped the cytolysin locus on one of these plasmids, pAD1, to an 8-kb region that was sufficient to impart cytolysin activity to *E. coli* (Ike *et al*., 1990). Sequencing of this locus revealed that it contained a six-gene operon (Segarra *et al*., 1991; Coburn *et al*., 1999).

More recently, the cytolysin operon has been observed to be encoded on the chromosome (Ike and Clewell, 1992; Shankar *et al*., 2002). An isolate from a mid-1980s hospital ward outbreak, and a second representing the first vancomycin resistant isolate in the United States, were both found to harbor a chromosomal copy of the cytolysin operon (Shankar *et al*., 2002). In these strains, the cytolysin operon was linked to other virulence genes within a stereotypical pathogenicity island (Shankar *et al*., 2002). While this pathogenicity island appears to be stably integrated into the chromosome, the cytolysin operon itself is subject to deletion at a frequent rate. A precise excision of a 17,036 basepair fragment of DNA that includes the 3' half of the cytolysin operon and six more ORFs, including the virulence gene *esp*, occurs at a frequency of 1 in $10^3$ during overnight *in vitro* cultivation (Shankar *et al*., 2002). This frequency is consistent with that of phase variation in other bacterial species (Fierer and Guiney, 2001). Thus, the cytolysin operon is a variable feature of the *E. faecalis* genome and may be lost or gained, thereby modulating virulence.

## THE MOLECULAR COMPONENTS OF CYTOLYSIN

The cytolytic phenotype requires the activity of at least five different proteins, all of which are encoded in the cytolysin operon (Segarra *et al*., 1991; Gilmore *et al*., 1994). The sixth gene in the cytolysin operon encodes an immunity factor that protects the toxin-producing cell from the toxin (Coburn *et al*., 1999).

The first and second genes of the operon, $cylL_L$ and $cylL_S$, encode the structural components of the toxin: two different peptide subunits of slightly different sizes. $CylL_L$, the larger subunit, and $CylL_S$, the smaller subunit, are initially synthesized as precursor peptides of 68 and 63 residues, respectively (Segarra *et al*., 1991). Each peptide must undergo a battery of posttranslational modifications, including residue dehydration, intra-peptide thioether bond formation, secretion, and two separate steps of proteolytic processing, before becoming active (Booth *et al*., 1996). Postmodification, $CylL_L$ is 38 amino acids and 3.44 KDa (this active form is designated $CylL_L$"), and $CylL_S$ is 21 amino acids and 2.03 KDa (designated $CylL_S$ ") (Segarra *et al*., 1991; Booth *et al*., 1996).

Amino acid analysis of the secreted forms of the peptides demonstrated that two of the three serines in $CylL_L$", the one serine in $CylL_S$", four of the five threonines in $CylL_L$", and all three threonines in $CylL_S$" were dehydrated (Booth *et al*., 1996). Thioether bond formation between a dehydrated serine or threonine and a local cysteine side chain results in the formation of a lanthionine or a β-methyllanthionine residue. Two such residues were detected in $CylL_L$", one in $CylL_S$" (Booth *et al*., 1996). The threonines encoded by codon 31 of $cylL_L$ and codon 42 of $cylL_S$ turned out to be the N terminal residue of each peptide, following complete processing to the mature peptides, and both were predicted to convert to 2-oxo-butyrines, following dehydration based on comparison to related toxins (Kellner *et al*., 1989; Sahl *et al*., 1995) and the observation of a hard block to Edman degradation at these residues (Booth *et al*., 1996). Other yet uncharacterized posttranslational modifications may also take place since the noted ones do not fully account for the discrepancy in predicted molecular weight based on DNA sequence and the mass spectroscopy measurements (Booth *et al*., 1996).

The properties of the structural subunits, which are small, cysteine-rich with dehydrated serines and threonines, lanthionine derivatization, hydrophilic N-termini, and hydrophobic carboxy-termini, classify them as members of the family of lantibiotic bacteriocins synthesized by a number of Gram-positive bacteria. The overall structure of the operon also resembles that of other lantibiotic-encoding loci. Thus, the *E. faecalis* cytolysin has been classified as a Type-A, lactocin S-subtype, pore-forming lantibiotic (Sahl *et al*., 1995). For several years, the *E. faecalis* cytolysin was the only known two-component lantibiotic, but others have since been identified, such as lactacin 3,147 from *Lactococcus lactis* (Ryan *et al*., 1996; Ryan *et al*., 1999) and staphylococcin C55 from *Staphylococcus aureus* (Navaratna *et al*., 1998; Navaratna *et al*., 1999).

The posttranslationally modified forms of $CylL_L$ and $CylL_S$ are designated $CylL_L^*$ and $CylL_S^*$, respectively. The modifications appear to be catalyzed directly or indirectly by a protein called CylM, encoded by the third gene in the cytolysin operon, *cylM*. CylM shares sequence similarity with a class of proteins involved in lantibiotic biosynthesis (Gilmore *et al*., 1994; Sahl *et al*., 1995) and mutations in *cylM* abolish cytolytic activity (Gilmore *et al*., 1994). Studies that probe the role and mechanism of the CylM protein more directly have not been done.

Export of $CylL_L^*$ and $CylL_S^*$ depends on the fourth gene in the operon, *cylB*, that encodes an ABC transporter protein of the HlyB family (Gilmore *et al*., 1990). Mutations that disrupt the production or folding of the CylB protein prevent externalization of both toxin subunit precursors, while mutations predicted to truncate CylB just before the C-terminal ATP-binding domain block only the secretion of $CylL_L^*$, not of $CylL_S^*$ (Gilmore *et al*., 1990; Gilmore *et al*., 1994). These results suggest that secretion of $CylL_L^*$ is an energy-dependent process, while secretion of $CylL_S^*$ depends on a concentration gradient. The CylB protein is predicted to contain an N-terminal cysteine protease domain, which is conserved among other dedicated transporters of peptide bacteriocins. In the case of LagD, the ABC transporter that exports the peptide precursors of the *Lactococcus lactis* bacteriocin lactacin 3,147, this protease domain was shown to cleave the leader sequence from the toxin precursor peptides (Havarstein *et al*., 1995). Given the sequence similarity between LagD and CylB, and also within the leader sequences of the *L. lactis* bacteriocin peptides and those of $CylL_L$ and $CylL_S$, it is presumed that CylB serves a homologous function to LagD, specifically the proteolytic cleavage of $CylL_L^*$ and $CylL_S^*$ concomitant with export. This first proteolytic processing step trims 24 residues from $CylL_L^*$ and 36 residues from $CylL_S^*$ (Booth *et al*., 1996). The cleavages take place within a stretch of 26 amino acids that are highly conserved (25 residues are identical) between the two peptides, although this stretch is situated closer to the N terminus of $CylL_L$ and is more centrally located in $CylL_S$. Precursor peptides that have undergone posttranslational modification, cleavage of the leader peptide, and secretion are designated $CylL_L'$ and $CylL_S'$.

$CylL_L'$ and $CylL_S'$ must each undergo an additional processing step before they are functional as a toxin. This extracellular activator, detected in the blood agar complementation analysis of Granato and Jackson (1969), is the CylA protein, a subtilisin-like serine protease encoded by the fifth gene in the operon, *cylA* (Segarra *et al*., 1991; Booth *et al*., 1996). CylA is synthesized as a pre-proenzyme whose secretion does not depend on *cylB*; rather, it is likely secreted via the general cellular secretion machinery (Segarra *et al*., 1991). Functional studies with purified CylA indicate that it removes the six N-terminal amino acids from both $CylL_L'$ and $CylL_S'$, cleaving within what remains of the stretch of amino acids conserved between the two subunits. The products of the CylA cleavages are the active lytic subunits, $CylL_L$" (38 amino acids) and $CylL_S$" (21 amino acids) (Booth *et al*., 1996). These interact in an unknown way to create a pore in the target cell membrane. The peptide sequence and processing sites of $CylL_L$ and $CylL_S$ are depicted in Figure 41.1.

The immunity factor encoded by the most 3' gene in the operon is called CylI. Sequence analysis of the 327-codon *cylI* ORF predicts a putative signal peptide,

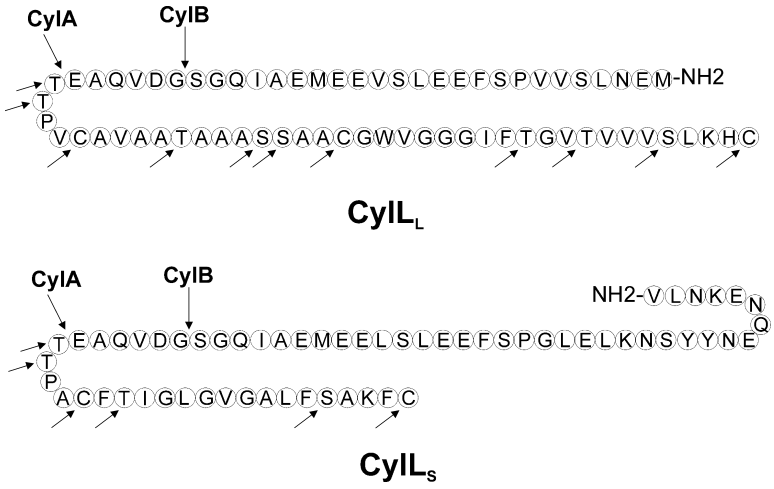



**FIGURE 41.1** The primary amino acid sequence of the cytolysin subunits $CylL_L$ and $CylL_S$. $CylL_L$ and $CylL_S$ are synthesized as 68 and 63 amino acid precursors. CylB cleaves leader sequences from each peptide as they are secreted. Following export, CylA proteolytically removes an additional six amino acids from the N-terminus of each subunit. The recognition sites for CylB and CylA cleavage are indicated by the labeled arrows, and the additional arrows indicate amino acids for which there is evidence of modification, or which are hypothesized to be modified.

putative transmembrane domains, and a putative zinc-metalloprotease motif, but functional studies to verify these predictions and to determine the mechanism of immunity have not been completed.

## REGULATION OF CYTOLYSIN EXPRESSION

The promoter for the *cyl* operon has been identified by primer extension analysis and is characterized by canonical −10 and −35 boxes. Reverse transcription PCR analysis indicates that all six genes are polycistronic and no internal promoters have been detected within the operon (W. Haas and M.S.G., unpublished observations). *cylA* (along with *cylI*) had been thought to be transcribed independently of the *cyl* operon (Segarra *et al*., 1991; Coburn *et al*., 1999), but an appropriately placed promoter has eluded identification. Results leading to that supposition may have been artefacts of read-through transcription from other vector elements. Interestingly, quantification of messages by real-time PCR shows that the $cylL_L$ and $cylL_S$ transcripts are five- to sixfold more abundant than the *cylM*, *cylB*, *cylA*, and *cylI* transcripts. An internal terminator element located between the $cylL_S$ and *cylM* genes seems to limit the quantity of full-length transcripts, perhaps serving as a mechanism for generating an efficient ratio of structural elements to catalytic elements for toxin production (W. Haas and M.S.G., unpublished observations).

Investigations into the regulation of expression of the cytolysin operon *in trans* have recently revealed three interesting and unexpected regulatory features of the system. (i) The cytolysin promoter is largely repressed by the dual action of two novel proteins, CylR1 and CylR2. (ii) The mature, active form of the small toxin subunit $CylL_S$" induces expression of the cytolysin operon once it exceeds a threshold concentration. (iii) The mature, active form of the large toxin subunit $CylL_L$" inhibits the autoinducing activity of $CylL_S$" in the absence of target cells.

### Repression by CylR1 and CylR2

Immediately upstream of the *cyl* operon lies a two-gene operon in the opposite orientation that is divergently transcribed from a promoter that overlaps with the *cyl* operon promoter (Haas *et al*., 2002). Using a *lacZ* fusion, experiments monitoring activity of the cytolysin operon promoter $P_{lys}$ demonstrated that transcription was significantly reduced when *cylR1* and *cylR2* were concomitantly expressed in the same cell (Haas *et al*., 2002). The promoter-proximal of these regulatory genes was named *cylR1,* and the promoter-distal gene named *cylR2*. Mutations in either *cylR1* or *cylR2* or both resulted in full derepression of the cytolysin promoter (Haas *et al*., 2002).

The 66 amino acid CylR2 protein has been purified, and both its x-ray crystal structure (at 1.9A resolution) and its NMR solution structure solved (Rumpel *et al*., 2004; Razeto *et al*., 2004). The structures show that CylR2 dimerizes and possesses a helix-turn-helix DNA-binding domain. Gel-shift analysis indicates that CylR2 binds specifically to a 22-basepair inverted repeat sequence that lies just upstream of the −35 box of the $P_{lys}$ promoter (Rumpel *et al*., 2004). In the presence of the inverted repeat DNA fragment, CylR2 residues undergo NMR chemical shift perturbations that corroborate the gel-shift results and implicate residues Q29, N40, and Q44 as particularly important for DNA binding. Accordingly, a $CylR2^{N40A}$ mutant fails to

repress a $P_{lys}$-*lacZ* fusion, furnishing strong evidence that CylR2 directly represses transcription from the $P_{lys}$ promoter (Rumpel *et al*., 2004).

CylR1 has not yet been purified. Sequence analysis indicates that it is 94 residues long with three predicted alpha-helical transmembrane domains (Haas *et al*., 2002). There are no close homologues in the GenBank database, and the mechanism by which it co-represses the $P_{lys}$ promoter along with CylR2 is yet unknown. As discussed below, CylR1 is presumed to play a role in transmitting the signal for induction of transcription of the *cyl* operon to the promoter-binding protein CylR2.

## Induction by $CylL_S$"

Repression of $P_{lys}$ by CylR1 and CylR2 is not absolute; a low basal level of transcription is still detected in the presence of both intact genes (Haas *et al*., 2002). This allows for the possibility that one or more *cyl* operon products could serve as a transcriptional autoinducer in a quorum-sensing capacity. That is, a threshold concentration of a gene product, achieved via basal-level transcription only when cell density is high, triggers increased transcription from its own promoter. Quorum-sensing autoinduction is employed by a number of pathogenic bacteria to ensure that virulence genes are only highly expressed when there are enough cells present to mount a successful infection (Miller and Bassler, 2001) (see Chapter 4). There is strong evidence that this is the case with induction of *cyl* operon expression. The mature, active form of the small toxin subunit $CylL_S$", encoded by the first gene in the operon, induced transcription from the $P_{lys}$ promoter when added to a culture of a $P_{lys}$-*lacZ* reporter strain at a sufficiently high concentration (Haas *et al*., 2002). Additionally, a $P_{lys}$-*lacZ* reporter strain that also harbored the native cytolysin operon did not express *lacZ* until the cell density in planktonic culture exceeded $10^7$ cells $mL^{-1}$. Only $CylL_S$" and not $CylL_S$' or any form of $CylL_L$ was found to have this autoinducing activity (Haas *et al*., 2002).

The mechanism by which $CylL_S$" effects derepression of $P_{lys}$ is unknown. A simple model is one in which $CylL_S$" interacts with the putative membrane-bound CylR1 protein at the cell surface, causing a signal transduction event that disrupts the repressive binding of CylR2 on the $P_{lys}$ promoter (Haas *et al*., 2002). However, this model is likely to prove too simple, as the DNA binding site of CylR2 is predicted to also block transcription from the divergent promoter ($P_R$) of the *cylR1R2* operon (Rumpel *et al*., 2004), and yet $CylL_S$" does not induce transcription of *cylR1* or *cylR2* concomitantly with transcription of the *cyl* operon (Haas *et al*., 2002).

## Target cell sensing by $CylL_L$"

As described above, a long unanswered puzzle about *E. faecalis* cytolytic activity had been E.W. Todd's observation that it could not be recovered from filtrates of cultures of the same strains that exhibited hemolytic activity on blood agar (Todd, 1934). However, if erythrocytes were included in the liquid cultures, *cyl*-dependent hemolytic activity was observed (Coburn *et al*., 2004). Together, these results suggested that there might be a mechanism to prevent cytolysin expression in the absence of target cells and to trigger it in their presence.

A first hint that $CylL_L$" might be a target cell sensor came from experiments examining $CylL_S$"-inducing activity. $CylL_S$"-dependent induction of *cyl* operon transcription was significantly greater in strains harboring a mutation in $cylL_L$ that truncated $CylL_L$' than in a wild-type strain (Haas *et al*., 2002; Coburn *et al*., 2004). The ability of $CylL_L$" to inhibit the inducing activity of $L_S$" was confirmed by experiments with purified $CylL_L$" and $CylL_S$" demonstrating that (i) $CylL_L$" blocks $CylL_S$"-mediated induction of a $P_{lys}$-*lacZ* reporter strain when the two components are present in equimolar concentration, and (ii) when mixed in solution $CylL_L$" and $CylL_S$" form inert, stable, SDS-resistant multimers ranging in apparent size from 132 kDa to over 216 kDa (Coburn *et al*., 2004).

A model proposing that $CylL_L$" prevents $CylL_S$"-mediated induction in the absence of target cells, but not in their presence, emerged from experiments demonstrating that pre-incubating $CylL_L$" with erythrocytes attenuates its ability to inhibit $CylL_S$"-mediated induction of the *cyl* operon (Coburn *et al*., 2004). The ability of $CylL_L$" to influence the activity of $CylL_S$" based on the presence of target cells depends on the ability of $CylL_L$" to absorb or bind to a target cell membrane differently than $CylL_S$". Evidence that this is indeed the case is derived from surface plasmon resonance experiments demonstrating that $CylL_L$" binds to lipid bilayers with more than a sixfold greater affinity than $CylL_S$". Furthermore, the kinetics of hemolysis are greatly increased if $CylL_L$" is preincubated with erythrocytes prior to addition of $CylL_S$", but are delayed if the subunits are added in the opposite order (Coburn *et al*., 2004).

Taken together, the experimental results support a model in which the structural subunits of the cytolysin toxin are also centrally involved in regulating toxin expression, such that the cell does not waste energy synthesizing and processing toxin unless two important criteria are met: Bacterial cell density is high and target cells are present. This model is illustrated in Figure 41.2.

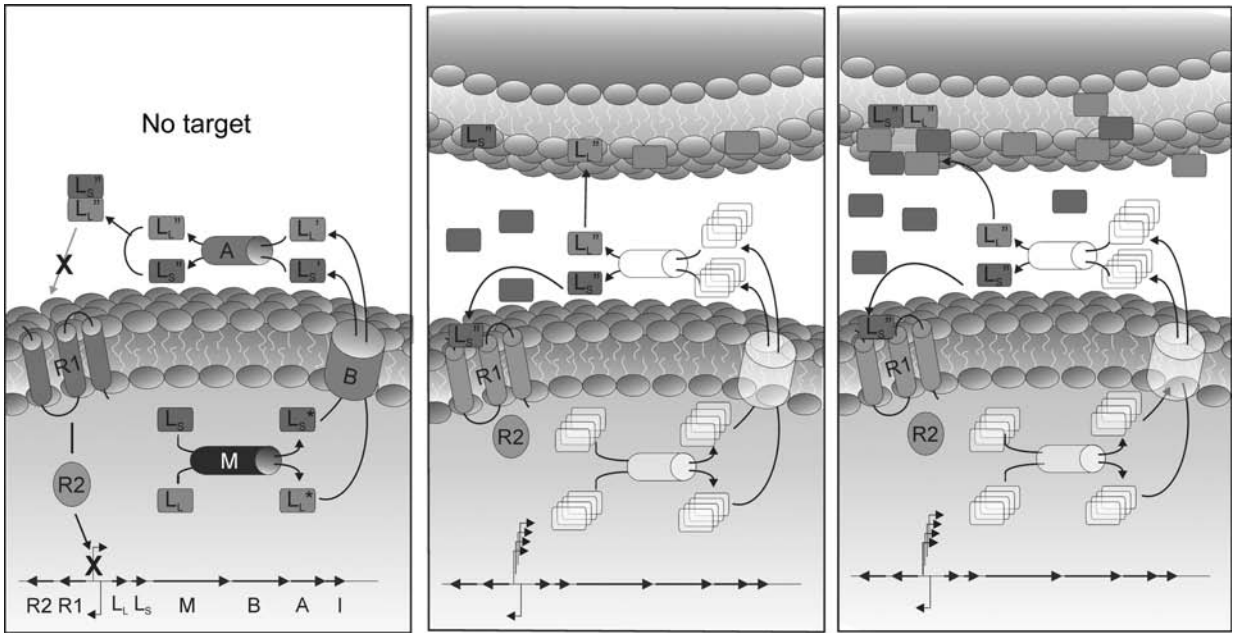



**FIGURE 41.2** Model for the expression of the *Enterococcus faecalis* cytolysin that involves quorum sensing of the inducer $CylL_S$ ", and the detection of target cells by $CylL_L$ ". In the absence of a target cell (left panel), $CylL_L$ " and $CylL_S$", as well as the gene products necessary for post-translational modification (CylM), secretion (CylB), extracellular activation (CylA), and producer immunity (CylI), are expressed at basal levels. The mature cytolysin subunits interact to form an inactive complex that is incapable of affecting target cell lysis and lacks the ability to induce high-level expression of the cytolysin operon. In the presence of a target cell (middle panel), $CylL_L$" binds preferentially to the target cell to the apparent exclusion of $CylL_S$", which allows free $CylL_S$ " to accumulate to a level necessary to trigger high-level cytolysin expression, hypothetically by altering the behavior of the CylR1 and CylR2 transcriptional repressors. Accumulation of $CylL_S$" and $CylL_L$" leads to target cell lysis (right panel) presumably by the formation of a pore.

## MOLECULAR MECHANISM OF CYTOLYSIN ACTIVITY

Little is known about the mechanistic details of cytolysin toxin subunit interactions with each other and with target cell membranes. It is not clear what properties of the cytolysin toxin uniquely endow it with the ability to target both Gram-positive bacteria and some, but not all, mammalian erythrocytes. A relatively higher phosphotidylcholine content in the membranes of erythrocytes most susceptible to cytolysin suggested a role for this lipid in targeting (Roelofsen *et al*., 1964). Support for this hypothesis was furnished by the observation that phosphatidlycholine inhibited the cytolysin-mediated lysis of horse erythrocytes (Miyazaki *et al*., 1993). However, demonstration of a direct interaction or mechanistic basis for this observation is still lacking.

Bacterial cell membranes also competitively inhibit cytolysin activity, and they do so more effectively (by three orders of magnitude) than eukaryotic cell membranes (Basinger and Jackson, 1968). These data suggest that cytolysin may recognize a more specific receptor molecule on bacterial cell membranes than on eukaryotic membranes. The lantibiotic nisin produced by *Lactococcus lactis* uses the bacterial cell wall precursor lipid II as a docking molecule to initiate contact with the cytoplasmic membrane (Brotz *et al*., 1998; Wiedemann *et al*., 2004). It is possible that *E. faecalis* cytolysin employs a similar docking molecule. The variable spectrum of susceptibility of bacterial cells to cytolysin may be due to variable levels of a docking molecule and/or variable levels of an inhibitory factor present within the cell wall or membrane. Both lecithin and ribitol teichoic acid esterified to D-alanine have been found to inhibit cytolytic activity (Brock and Davie, 1963; Davie and Brock, 1965). The resistance of Gram-negative bacteria to cytolysin is probably attributable to the outer membrane, since L-forms of certain species were susceptible (Kalmanson *et al*., 1970).

Thus, the properties of cell membranes that render them cytolysin-senstive are still vaguely defined. Evidence that the cytolysin toxin actually forms a pore in the target cell membrane is derived from parallels drawn with other lantibiotics and hemolysins, and from the observation that cytolysin effects the release of carboxyfluorescein from liposomes (C.P. Bogie and M.S.G., unpublished observations).

Recently a structure-function study of the small toxin subunit was carried out in which every residue of the mature $CylL_S$" protein was individually changed to alanine. Each of the resulting 18 mutants was tested for its molecular weight, hemolytic activity, bacteriocin activity, and *cyl* operon-inducing activity. The majority of the residues appeared to be important for effective hemolytic activity. However, several of the mutations that blocked hemolytic activity did not impair bacteriocin activity. These results suggest that the mechanism of hemolytic activity is in part distinct from that of bacteriocin activity. The residues important for autoinducing activity also appear to be distinct from those required for either hemolytic or bacteriocin activity, as several of the residues that blocked hemolytic activity did not impair inducing activity, and one of the changes, G51A, that blocked inducing activity did not impair bacteriocin activity (P. Coburn and M.S.G., unpublished observations). Whether the distinctive mechanistic requirements for each of the residues is related to specific contacts with other proteins, contacts with lipids, or the overall physical properties of the peptide has yet to be determined.

Interestingly, several of the mutations found to block hemolytic activity actually enhanced bacteriocin and/or induction activity (P. Coburn and M.S.G., unpublished observations). Apparently, maximal bacteriocin/induction capabilities were not selected for evolutionarily. The mutant analysis suggests that these may have been compromised to enable hemolytic activity or some other toxin behavior that may have provided a competitive advantage in obtaining nutrients, thwarting predators, or colonizing hosts. A second, and not mutually exclusive possibility, is that suboptimal bacteriocin/induction activities were selected to prevent self-killing. The ability of *cylI* to protect *E. faecalis* against the most potent bacteriocin mutant has not yet been tested. One of the mutations, P45A, enhances all three assayed activities—hemolysis, bacterial killing, and induction (P. Coburn and M.S.G., unpublished observations). Proline may have been selected at this position to facilitate self-protection (a testable hypothesis), to facilitate lysis of another cell type that has not yet been assayed, to stabilize the molecule against proteases in the environment, or to carry out another activity of $CylL_S$" that has not yet been discovered.

For a number of the mutants, MALDI-TOF analysis failed to detect a peptide from the culture supernatant that was within the predicted molecular weight range of the mutant peptide (P. Coburn and M.S.G., unpublished observations). The failure to detect predicted peptide products for T43A, T44A, C47A, T49A, G55A, S59A, and K61A may prove to be biologically informative. There is evidence that T43, T44, T49, and S59 are all posttranslationally modified. At least one is probably involved in the formation of a lanthionine or β-methyllanthionine residue (Booth *et al*., 1996), and C47 may also be involved in the formation of that residue. Studies of other lantibiotics have shown that the formation of the thioether bridges of lanthionine derivatives confers stability and protease resistance to the peptides (de Vuyst and Vandamme, 1994; Bierbaum *et al*., 1996). Nothing is known specifically about what roles G51 or K61 might play, but K61 represents the only charged residue in the entire mature peptide. Instability, secretion defects, and processing defects are possible explanations for why these mutant peptides were not detected in the culture supernatants by MALDI-TOF. Ongoing studies will address these questions, and a similar analysis of $CylL_L$ mutants may be carried out.

## CYTOLYSIN AS A COLONIZATION AND VIRULENCE FACTOR

The similarities between the cytolysin toxin and the operon encoding it to the toxins and operons of the members of the lantibiotic class of bacteriocins suggest that the earliest incarnation of the cytolysin operon conferred a competitive advantage to *E. faecalis* over other Gram-positive strains vying for control of the same niche. This hypothesis is supported by an *in vitro* experiment in which a cytolytic strain outcompeted a bacteriocin-sensitive strain in mixed liquid culture even when the sensitive strain started out in 100-fold excess (Brock and Davie, 1963). A selective advantage for the ability to fend off protozoan or nematode predators, obtain more nutrients, and/or colonize more niches within host animals may have driven the evolution from a lantibiotic to a cytolysin also capable of lysing eukaryotic cells. However, the fact that the cytolysin determinants are not present in all strains and can in fact become excised at a high frequency (Shankar *et al*., 2002) suggests that toxin activity may not confer a selective advantage to cells in all niches or circumstances.

Does cytolysin toxin contribute to the virulence of *E. faecalis* strains in animal hosts? While many infections are established with non-cytolytic strains, analysis of clinical isolates in several studies supports the hypothesis that cytolysin enhances the severity of infection and may also enhance the propensity to infect. A study examining clinical isolates from a Japanese hospital in the 1980s determined that approximately 60% were cytolytic, compared to 17% of isolates from the stools of

healthy students at a nearby medical school (Ike *et al*., 1987). *E. faecalis* isolates from endopthalmitis patients in the United States were also found to be enriched for the cytolytic phenotype—46.5% versus the 0–17% typically found in stools of healthy volunteers (Booth *et al*., 1998). A number of studies also indicate enrichment (25–28%) for the cytolytic phenotype among isolates from urinary tract infections (Rantz and Kirby, 1943; Facklam, 1972; Libertin *et al*., 1992). On the other hand, isolates from endocarditis patients show a much lower incidence of a cytolytic phenotype (11–16%) (Facklam, 1972; Coque *et al*., 1995; Huycke *et al*., 1995).

A study of bacteremia patients in a Wisconsin hospital and their *E. faecalis* blood isolates determined that patients infected with cytolytic, gentamicin-resistant strains were at a fivefold increased risk of death within three weeks of their bacteremia compared to patients with non-cytolytic, gentamicin-susceptible strains (Huycke *et al*., 1991). The contribution of antibiotic resistance was discounted from this statistic, since all strains remained susceptible to one or more antibiotics, and the disease outcome was not associated with treatment modality. On the other hand, a separate study of bacteremia patients involved in a multicenter study (Vergis *et al*., 2001) determined that only 11% of isolates were cytolytic, and while 9% of patients infected with cytolytic strains died, there was no significant association between 14-day mortality and the cytolytic phenotype, either alone or in combination with *gelE* or *esp*, two other *E. faecalis* virulence genes (Vergis *et al*., 2002).

Cytolysin is one among multiple factors of the bacterium and host that determine *E. faecalis* pathogenicity. Since epidemiological analyses are limited to correlations and are riddled with a wide range of uncontrolled variables, studies in which animal models are infected with isogenic cytolytic and non-cytolytic strains are informative for quantifying the effect of virulence traits on an infected host. The results of such studies uniformly support a key role for cytolysin in *E. faecalis* virulence. In two separate studies, intraperitoneal infections of mice with a cytolytic strain of *E. faecalis* caused a significantly greater rate of lethality than infections with a non-cytolytic strain, with approximately an order of magnitude difference in the $LD_{50}$ (Ike *et al*., 1984; Dupont *et al*., 1998). In one of the studies, a strain with increased copies of the cytolysin operon lowered the $LD_{50}$ even further (Ike *et al*., 1984). A rabbit endocarditis model also indicated an important role for cytolysin as a determinant of lethality. In this study, isogenic *E. faecalis* strains harboring the cytolysin operon, or the gene for aggregation substance (a surface protein involved in conjugation and surface attachment), or both, were injected intravenously after catheterization. Infection with strains expressing only cytolysin did not result in lethality, but cytolysin synergized with aggregation substance to cause lethality in 55% of infected animals versus only 15% of animals infected with a strain expressing just aggregation substance (Chow *et al*., 1993).

Enhanced virulence due to cytolysin was also clear from the development of a rabbit endopthalmitis model system (Jett *et al*., 1992). This system takes advantage of the immune privilege of the eye that enables establishment of an infection with 10–100 bacterial cells. In some ways, this mode of infection is physiologically more similar to nosocomially acquired infections in unhealthy human hosts than is the injection of large numbers of cultured bacteria into healthy animals. Moreover, the progression of a single eye infection can be monitored over multiple time points using the standard tools of ophthalmology. Infection of rabbit eyes with 100 cytolytic *E. faecalis* cells caused the destruction of retinal tissue and architecture and a 99% reduction in retinal neuro-responsiveness. Conversely, infection of rabbit eyes with 100 non-cytolytic *E. faecalis* cells left the retinal tissues largely intact and reduced retinal function by 74.2% (Jett *et al*., 1992).

A non-mammalian host, the nematode *Caenorhabiditis elegans* that ingests *E. faecalis*, is also sensitive to cytolysin. Worms fed a cytolytic strain are killed faster than worms fed a non-cytolytic strain (Garsin *et al*., 2001). While these results suggest that targets of cytolysin might be ubiquitous, cytolysin did not seem to aggravate symptoms in a rat peritonitis model in which the symptoms caused by any *E. faecalis* strain were mild to begin with (Dupont *et al*., 1998). The basis for the resistance of rats to *E. faecalis* and cytolysin are unknown and highlight the importance of carefully interpreting results from animal models.

There are a number of ways in which the presence of the cytolysin toxin might aid establishment of infection and enhance disease severity. It can lyse erythrocytes, and there is evidence that it can damage neural tissue (Jett *et al*., 1992; Stevens *et al*., 1992; Jett *et al*., 1995) and intestinal epithelial tissue (P. Coburn and M.S.G., unpublished observations). These injuries may provide more nutrients for the bacteria, undermine the health of the host, and/or enable the bacteria to penetrate deeper into uncolonized tissue. Cytolysin has also been shown to be active against macrophages and polymorphonuclear leukocytes (Miyazaki *et al*., 1993), suggesting that it could weaken the host immune response. Interestingly though, a cytolytic strain did not maintain viability within mouse peritoneal macrophages any longer than did an isogenic non-cytolytic strain (Gentry-Weeks *et al*., 1999). It is also

possible that the bacteriocin activity of the cytolysin could destablilize the commensal gut flora, clearing the way for colonization by cytolytic *E. faecalis*. However, there is no evidence for this, and in fact the competitive advantage of a cytolytic strain over a non-cytolytic strain observed *in vitro* was not recapitulated *in vivo* in an experiment in which mice pretreated with antibiotics were fed a one-to-one mixture of the two strains. The ratio of the two strains recovered from stool samples one and seven days later hovered at one-to-one (Huycke *et al*., 1995). It is unknown to what extent this experiment reflects the events within a human GI tract, especially considering that the cytolytic strain was isolated from a human patient, and there is evidence that strains of *E. faecalis* are specifically adapted to their host species (Willems *et al*., 2000).

## CONCLUSION

In summary, the cytolysin toxin of *E. faecalis* is a two-subunit hemolysin and bacteriocin capable of lysing a broad range of target cells. The maturation process of the two structural toxin subunits is similar to the maturation process of other lantibiotic bacteriocins, but the regulatory activity of each of these subunits in calibrating toxin expression to reflect cell density and the presence of target cells is novel. Further studies of the components of the cytolysin locus and the target cell-toxin interactions are certain to yield more interesting mechanisms and may inform the development of a therapeutic agent to neutralize this virulence factor.

## ACKNOWLEDGMENTS

We thank Phillip S. Coburn and Christopher M. Pillar for helpful advice during the preparation of this manuscript. Portions of the research described in this manuscript were supported by NIH grants EY08289 and AI41108, and by an unrestricted award from Research to Prevent Blindness.

## REFERENCES

Basinger, S.F., Jackson, R.W. (1968). Bacteriocin (hemolysin) of *Streptococcus zymogenes. The Journal of Bacteriology* **96,** 1895–1902.

Bierbaum, G., Szekat, C., Josten, M., Heidrich, C., Kempter, C., Jung, G. and Sahl, H.G. (1996). Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. *Appl.Environ.Microbiol*. **62,** 385–392.

Booth, M.C., Bogie, C.P., Sahl, H.G., Siezen, R.J., Hatter, K.L. and Gilmore, M.S. (1996). Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. *Mol. Microbiol*. **21,** 1175–1184.

Booth, M.C., Hatter, K.L., Miller, D., Davis, J., Kowalski, R., Parke, D.W., Chodosh, J., Jett, B.D., Callegan, M.C., Penland, R. and Gilmore, M.S. (1998). Molecular epidemiology of *Staphylococcus aureus* and *Enterococcus faecalis* in endophthalmitis. *Infect. Immun*. **66,** 356–360.

Brock, T.D. and Davie, J.M. (1963). Probable identity of a group D hemolysin with a bacteriocine. *J. Bacteriol*. **86,** 708–712.

Brock, T.D., Peacher, B. and Pierson, D. (1963). Survey of the bacteriocins of enterococci. *The Journal of Bacteriology* **86,** 702–707.

Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G. and Sahl, H.G. (1998). Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin, and other lantibiotics. *Mol. Microbiol*. **30,** 317–327.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B. and Zervos, M.J. (1993). Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrob. Agents Chemother*. **37,** 2474–2477.

Clewell, D.B. (1993). Bacterial sex pheromone-induced plasmid transfer. *Cell* **73,** 9–12.

Clewell, D.B., Tomich, P.K., Gawron-Burke, M.C., Franke, A.E., Yagi, Y. and An, F.Y. (1982). Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. *The Journal of Bacteriology* **152,** 1220–1230.

Coburn, P.S., Hancock, L.E., Booth, M.C. and Gilmore, M.S. (1999). A novel means of self-protection, unrelated to toxin activation, confers immunity to the bactericidal effects of the *Enterococcus faecalis* cytolysin. *Infect. Immun*. **67,** 3339–3347.

Coburn, P.S., Pillar, C.M., Jett, B.D., Haas, W. and Gilmore, M.S. *Enterococcus faecalis* sense target cells and in response expresses cytolysin. *Science* **306,** 2270.

Coque, T.M., Patterson, J.E., Steckelberg, J.M. and Murray, B.E. (1995). Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. *J. Infect. Dis*. **171,** 1223–1229.

Davie, J.M. and Brock, T.D. (1965). Action of streptolysin S, the group D hemolysin, and phospholipase C on whole cells and spheroplasts. *The Journal of Bacteriology* **91,** 595–600.

de Vuyst, L. and Vandamme, E.J. (1994). Nisin, a lantibiotic produced by *Lactococcus Lactis* Subsp. *Lactis*: properties, biosynthesis, fermentation and applications. In: *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics, and Applications*, (eds. L. de Vuyst, E.J. Vandamme), pp. 151–200. Blackie Academic & Professional, London.

Dunny, G.M. and Clewell, D.B. (1975). Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. *The Journal of Bacteriology* **124,** 784–790.

Dunny, G.M. and Leonard, B.A. (1997). Cell-cell communication in Gram-positive bacteria. *Annu. Rev. Microbiol*. **51,** 527–564.

Dupont, H., Montravers, P., Mohler, J. and Carbon, C. (1998). Disparate findings on the role of virulence factors of *Enterococcus faecalis* in mouse and rat models of peritonitis. *Infect. Immun*. **66,** 2570–2575.

Elsner, H.A., Sobottka, I., Mack, D., Claussen, M., Laufs, R. and Wirth, R. (2000). Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. *Eur. J. Clin. Microbiol. Infect. Dis*. **19,** 39–42.

Facklam, R.R. (1972). Recognition of group D streptococcal species of human origin by biochemical and physiological tests. *Appl. Microbiol*. **23,** 1131–1139.

Fierer, J. and Guiney, D.G. (2001). Diverse virulence traits underlying different clinical outcomes of Salmonella infection. *J. Clin. Invest*. **107,** 775–780.

Garsin, D.A., Sifri, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B.E., Calderwood, S.B. and Ausubel, F.M. (2001). A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci. USA* **98,** 10892–10897.

Gentry-Weeks, C.R., Karkhoff-Schweizer, R., Pikis, A., Estay, M. and Keith, J.M. (1999). Survival of *Enterococcus faecalis* in mouse peritoneal macrophages. *Infect. Immun*. **67,** 2160–2165.

Gilmore, M.S. (1991). *Enterococcus faecalis* hemolysin/bacteriocin. In: *Genetics and Molecular Biology of Streptococci, Lactococci and Enterococci* (eds. G.M. Dunny, P.P. Cleary, L.L. McKay), pp. 206–213 American Society for Microbiology, Washington, D.C.

Gilmore, M.S., Coburn, P.S., Nallapareddy, S.R. and Murray, B.E. (2002). Enterococcal virulence. In: *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* (eds. M.S. Gilmore, D.B. Clewell, P. Courvalin, G.M. Dunny, B.E. Murray, L.B. Rice), pp. 301–354, ASM Press, Washington, D.C.

Gilmore, M.S., Segarra, R.A. and Booth, M.C. (1990). An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. *Infect. Immun*. **58,** 3914–3923.

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R. and Clewell, D.B. (1994). Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *The Journal of Bacteriology* **176,** 7335–7344.

Granato, P.A. and Jackson, R.W. (1969). Bicomponent nature of lysin from *Streptococcus zymogenes. J. Bacteriol*. **100,** 865–868.

Haas, W., Shepard, B.D. and Gilmore, M.S. (2002). Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. *Nature* **415,** 84–87.

Havarstein, L.S., Diep, D.B. and Nes, I.F. (1995). A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol. Microbiol*. **16,** 229–240.

Huycke, M.M., Gilmore, M.S., Jett, B.D. and Booth, J.L. (1992). Transfer of pheromone-inducible plasmids between *Enterococcus faecalis* in the Syrian hamster gastrointestinal tract. *J. Infect. Dis*. **166,** 1188–1191.

Huycke, M.M., Joyce, W.A. and Gilmore, M.S. (1995). *Enterococcus faecalis* cytolysin without effect on the intestinal growth of susceptible enterococci in mice. *J. Infect. Dis*. **172,** 273–276.

Huycke, M.M., Sahm, D.F. and Gilmore, M.S. (1998). Multiple drug-resistant enterococci: the nature of the problem and an agenda for the future. *Emerg. Infect. Dis*. **4,** 239–249.

Huycke, M.M., Spiegel, C.A. and Gilmore, M.S. (1991). Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis. Antimicrob. Agents Chemother*. **35,** 1626–1634.

Ike, Y. and Clewell, D.B. (1992). Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups, as well as by the chromosome. *The Journal of Bacteriology* **174,** 8172–8177.

Ike, Y., Clewell, D.B., Segarra, R.A. and Gilmore, M.S. (1990). Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn917 insertional mutagenesis and cloning. *The Journal of Bacteriology* **172,** 155–163.

Ike, Y., Hashimoto, H. and Clewell, D.B. (1984). Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. *Infect. Immun*. **45,** 528–530.

Ike, Y., Hashimoto, H. and Clewell, D.B. (1987). High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. *J. Clin. Microbiol*. **25,** 1524–1528.

Jacob, A.E., Douglas, G.J. and Hobbs, S.J. (1975). Self-transferable plasmids determining the hemolysin and bacteriocin of *Streptococcus faecalis* var. *zymogenes. The Journal of Bacteriology* **121,** 863–872.

Jett, B.D. and Gilmore, M.S. (1990). The growth-inhibitory effect of the *Enterococcus faecalis* bacteriocin encoded by pAD1 extends to the oral streptococci. *J. Dent. Res*. **69,** 1640–1645.

Jett, B.D., Huycke, M.M. and Gilmore, M.S. (1994). Virulence of enterococci. *Clin. Microbiol. Rev*. **7,** 462–478.

Jett, B.D., Jensen, H.G., Atkuri, R.V. and Gilmore, M.S. (1995). Evaluation of therapeutic measures for treating endophthalmitis caused by isogenic toxin-producing and toxin-nonproducing *Enterococcus faecalis* strains. *Invest. Ophthalmol. Vis. Sci*. **36,** 9–15.

Jett, B.D., Jensen, H.G., Nordquist, R.E. and Gilmore, M.S. (1992). Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infect. Immun*. **60,** 2445–2452.

Jones, M.E., Draghi, D.C., Thornsberry, C., Karlowsky, J.A., Sahm, D.F. and Wenzel, R.P. (2004). Emerging resistance among bacterial pathogens in the intensive care unit — a European and North American surveillance study (2000–2002). *Ann. Clin. Microbiol. Antimicrob*. **3,** 14.

Kalmanson, G.M., Hubert, E.G. and Guze, L.B. (1970). Effect of bacteriocin from *Streptococcus faecalis* on microbial L-forms. *J. Infect. Dis*. **121,** 311–315.

Kellner, R., Jung, G., Josten, M., Kaletta, C., Entian, K.-D. and Sahl, H.-G. (1989). Pep5, a new lantibiotic: structure elucidation and amino acid sequence of the propeptide. *Angew. Chem. Int. Ed. Engl*. **28,** 616–619.

Kobayashi, R. (1940). Studies concerning hemolytic streptococci: typing of human hemolytic streptococci and their relation to diseases and their distribution on mucous membranes. *Kitasato Arch. Exp. Med*. **17,** 218–241.

Libertin, C.R., Dumitru, R. and Stein, D.S. (1992). The hemolysin/bacteriocin produced by enterococci is a marker of pathogenicity. *Diagn. Microbiol. Infect. Dis*. **15,** 115–120.

Miller, M.B. and Bassler, B.L. (2001). Quorum sensing in bacteria. *Annu. Rev. Microbiol*. **55,** 165–199.

Miyazaki, S., Ohno, A., Kobayashi, I., Uji, T., Yamaguchi, K. and Goto, S. (1993). Cytotoxic effect of hemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. *Microbiol. Immunol*. **37,** 265–270.

Navaratna, M.A., Sahl, H.G. and Tagg, J.R. (1998). Two-component anti-*Staphylococcus aureus* lantibiotic activity produced by *Staphylococcus aureus* C55. *Appl. Environ. Microbiol*. **64,** 4803–4808.

Navaratna, M.A., Sahl, H.G. and Tagg, J.R. (1999). Identification of genes encoding two-component lantibiotic production in *Staphylococcus aureus* C55 and other phage group II *S. aureus* strains and demonstration of an association with the exfoliative toxin B gene. *Infect. Immun*. **67,** 4268–4271.

Noble, C.J. (1978). Carriage of group D streptococci in the human bowel. *J. Clin. Pathol*. **31,** 1182–1186.

Sherwood, N.P., Russell, B.E., Jay, A.R. and Bowman, K. (1949). Studies on streptococci. III. New antibiotic substances produced by beta hemolytic streptococci. *J. Infect. Dis*. **84,** 88–91.

Rantz, L.A. and Kirby, W.M.M. (1943). Enterococcic infections. An evaluation of the importance of fecal streptococci and related organisms in the causation of human disease. *Arch. Intern. Med*. **71,** 516–528.

Razeto, A., Giller, K., Haas, W., Gilmore, M.S., Zweckstetter, M. and Becker, S. (2004). Expression, purification, crystallization, and preliminary crystallographic studies of the *Enterococcus faecalis* cytolysin repressor CylR2. *Acta Crystallogr. D. Biol. Crystallogr*. **60,** 746–748.

Richards, M.J., Edwards, J.R., Culver, D.H. and Gaynes, R.P. (2000). Nosocomial infections in combined medical-surgical intensive care units in the United States. *Infect. Control Hosp. Epidemiol*. **21,** 510–515.

Roelofsen, B., De Gier, J. and Van Deenen, L.L. (1964). Binding of lipids in the red cell membrane. *J. Cell. Comp. Physiol*. **63,** 233–243.

Rumpel, S., Razeto, A., Pillar, C.M., Vijayan, V., Taylor, A., Giller, K., Gilmore, M.S., Becker, S. and Zweckstetter, M. (2004). Structure and DNA-binding properties of the cytolysin regulator CylR2 from *Enterococcus faecalis. EMBO J*. **23,** 3632–3642.

Ryan, M.P., Jack, R.W., Josten, M., Sahl, H.G., Jung, G., Ross, R.P. and Hill, C. (1999). Extensive posttranslational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. *J. Biol. Chem*. **274,** 37544–37550.

Ryan, M.P., Rea, M.C., Hill, C. and Ross, R.P. (1996). An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl. Environ. Microbiol*. **62,** 612–619.

Sahl, H.G., Jack, R.W. and Bierbaum, G. (1995). Biosynthesis and biological activities of lantibiotics with unique posttranslational modifications. *Eur. J. Biochem*. **230,** 827–853.

Segarra, R.A., Booth, M.C., Morales, D.A., Huycke, M.M. and Gilmore, M.S. (1991). Molecular characterization of the *Enterococcus faecalis* cytolysin activator. *Infect. Immun*. **59,** 1239–1246.

Shankar, N., Baghdayan, A.S. and Gilmore, M.S. (2002). Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis. Nature* **417,** 746–750.

Stark, J.M. (1960). Antibiotic activity of hemolytic enterococci. *Lancet* **i,** 733–734.

Stevens, S.X., Jensen, H.G., Jett, B.D. and Gilmore, M.S. (1992). A hemolysin-encoding plasmid contributes to bacterial virulence in experimental *Enterococcus faecalis* endophthalmitis. *Invest. Ophthalmol. Vis. Sci*. **33,** 1650–1656.

Todd, E.W. (1934). A comparative serological study of streptolysins derived from human and from animal infections, with notes on pneumococcal hemolysin, tetanolysin, and staphylococcus toxin. *J. Pathol. Bacteriol*. **39,** 299–321.

Vergis, E.N., Hayden, M.K., Chow, J.W., Snydman, D.R., Zervos, M.J., Linden, P.K., Wagener, M.M., Schmitt, B. and Muder, R.R. (2001). Determinants of vancomycin resistance and mortality rates in enterococcal bacteremia. a prospective multicenter study. *Ann. Intern. Med*. **135,** 484–492.

Vergis, E.N., Shankar, N., Chow, J.W., Hayden, M.K., Snydman, D.R., Zervos, M.J., Linden, P.K., Wagener, M.M. and Muder, R.R. (2002). Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis. Clin. Infect. Dis*. **35,** 570–575.

Wiedemann, I., Benz, R. and Sahl, H.G. (2004). Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. *The Journal of Bacteriology* **186,** 3259–3261.

Willems, R.J., Top, J., van Den, B.N., van Belkum, A., Endtz, H., Mevius, D., Stobberingh, E., van Den, B.A. and van Embden, J.D. (2000). Host specificity of vancomycin-resistant *Enterococcus faecium. J. Infect. Dis*. **182,** 816–823.

CHAPTER

# 42

# Streptolysin S: one of the most potent and elusive of all bacterial toxins

*Joyce C. S. de Azavedo, Kowthar Y. Salim, and Darrin J. Bast*

## INTRODUCTION

Streptolysin S (SLS) is responsible for the hallmark β-hemolytic phenotype seen around colonies of Group A *Streptococcus* (GAS), also known as *Streptococcus pyogenes*, when cultured on blood agar. By contrast, streptolysin O (SLO), another cytolysin produced by GAS, is oxygen-labile and thus only observed under anaerobic culture conditions. Although SLS was discovered in the 1930s (Todd, 1938; Weld, 1934), it has proven difficult to characterize due to the difficulty of obtaining purified preparations since additional factors are required for synthesis and a carrier molecule is needed to provide stability in solution. Consequently, it was not until 1986 that Loridan and Alouf first purified carrier-free SLS and were able to determine its molecular weight (Loridan and Alouf, 1986). However, attempts at N-terminal sequencing of the peptide proved to be unsuccessful, and the amino acid structure remains a mystery despite more recent genetic information as to the structural gene for SLS. An operon associated with SLS production has been identified (Betschel *et al*., 1998; Nizet *et al*., 2000). However, the functions of individual genes in the operon have not been elucidated, and furthermore the sequence of the proposed structural gene does not entirely agree with the published composition.

Although the cytolytic capacity of SLS has been well documented, its contribution to the pathogenesis of GAS disease was not clear until the use of SLS-deficient mutants demonstrated a role in lesion formation and tissue necrosis (Betschel *et al*., 1998). Recent work also provides evidence that the SLS RNA transcript might act as a regulator for other GAS determinants (Li *et al*., 1999; Mangold *et al*., 2004), in which case its role could prove to be complex. Its importance among pathogenic streptococci is underlined by the fact that it is also produced by Lancefield groups C and G streptococci (GGS and GCS) and has been recently reported in the zoonotic pathogen *Streptococcus iniae* (Flanagan *et al*., 1998; Fuller *et al*., 2002; Humar *et al*., 2002). Several detailed reviews have been published on the biological and physicochemical properties of SLS, as well as on the genetics of its production and its role in disease (Ginsburg, 1970; Alouf, 1980; Ginsburg, 1999; Nizet, 2002). In this review, we summarize early work and focus on the more recent findings, such as its genetic organization, regulation, and possible role(s) in the pathophysiology of streptococcal infections.

## GENERAL PROPERTIES AND PRODUCTION OF SLS

The zone of β-hemolysis surrounding SLS-producing streptococcal colonies on blood agar media is reflective of its cytolytic action. It is one of the most potent bacterial cytotoxins known, and it has been shown to exhibit highly toxic membrane-damaging effects on a variety of mammalian cell types, including red blood cells,

lymphocytes, polymorphonuclear leukocytes, platelets, tissue culture cells, and tumor cells (Ginsburg, 1970). It also disrupts subcellular membrane-bound organelles such as lysosomes, nuclei, and mitochondria (see Alouf, 1980).

SLS is synthesized *de novo* and while the majority of the toxin is cell bound, likely linked to the cell wall by lipoteichoic acid, cytoplasmic and extracellular forms have also been described (Calandra and Cole, 1981; Theodore and Calandra, 1981). The formation of SLS by GAS in culture was shown to occur during the stationary phase of growth in the presence of maltose (Ginsburg *et al*., 1963; Taketo and Taketo, 1964). However, the stability of the toxin in solution required the addition of "carrier" molecules or "inducers" such as albumin, α-lipoprotein, or yeast RNA (RNase-free core) (Alouf, 1980; Ginsburg, 1970). Interestingly, not only were the carriers chemically unrelated, but nonionic detergents such as Tween and Triton-X could also suffice (Ginsburg, 1970). Taken together, these data show that maltose is a requirement for the intracellular synthesis of SLS, but that a carrier is needed for its extracellular release. In addition to a carrier, it was shown that a bovine serum albumin derivative consisting of three peptide chains composed primarily of aspartic acid, glutamic acid, and leucine linked by two disulfide bridges was also necessary for release of cell-bound SLS (Akao *et al*., 1992). More recent studies using synthetic peptides have shown that it is the disulfide bridges that are essential, regardless of the number and types of amino acids that constitute the peptides (Akao *et al*., 1999).

Aniline dyes, such as Trypan Blue and Congo Red, abolish the action of SLS but not that of SLO or other hemolytic toxins (Alouf, 1980; Ginsburg, 1970). However, the mechanism of this phenomenon is not known. SLS activity is also inactivated by chymotrypsin, papain, ficin, and cathepsin but not by trypsin, pepsin, lysozyme, hyaluronidase, lipase, or muramidase (Ginsburg, 1970).

## BIOCHEMISTRY OF SLS

The first evidence that SLS was protein in nature was demonstrated by Koyama and Egami in 1963, who showed that a partially purified SLS-oligonucleotide mixture contained 12 different amino acids in varying molar ratios (Koyama and Egami, 1963). Using improved purification methods, Lai *et al.* in 1978 reported a 15-kDa protein containing 32 amino acids, of which 7.1 kDa was the associated carrier (Lai *et al*., 1978). It was estimated that the hemolytic moiety might be a 2.8-kDa peptide composed of approximately 28–30 amino acids.

It was not until 1986 that further modifications in purification methods eventually led to the identification of a 1.8-kDa peptide (pI 9.2), which was then thought to be SLS in its purest form (Loridan and Alouf, 1986; Alouf and Loridan, 1988). The SLS peptide was found to lack cysteine, histidine, proline, and tryptophan. Unfortunately, attempts to deduce amino acid sequence by Edman degradation failed, suggesting that the amino terminal may be blocked, or that the peptide moiety was cyclic (Alouf and Loridan, 1986). Interestingly, this finding at the protein level supports those at the genetic level and will be explained in more detail later in this review. Prior to the studies by Loridan and Alouf (1986, 1988), the isolation of carrier-free SLS in its native form had not been possible, given the strong interaction between the SLS peptide and the carrier.

## MECHANISM OF ACTION

Difficulties in obtaining homogeneous preparations of SLS, as well as the inability to raise antibodies against purified SLS, have limited studies on its mode of action. Although it is evident that SLS, like SLO, affects cell membranes to cause lysis, its precise mechanism of action is unclear, and it is unlikely that both streptolysins have the same mechanism of action. Cholesterol, which forms a major component of mammalian cell membranes, does not inhibit SLS activity, whereas several phospholipids, such as phosphatidyl choline and phosphatidyl ethanolamine, as well as sphingomyelin and lecithin, do (Elias *et al*., 1966). On the other hand, SLO binds to cholesterol giving rise to large transmembrane pores leading to lysis (Palmer *et al*., 1998). By comparison, there is evidence that SLS creates small pores in the membrane, stemming from the fact that sub-lytic concentrations of SLS cause a release only of low molecular weight molecules from human fibroblast cells in tissue culture (Thelestam and Mollby, 1979). Investigation of membrane damage caused by SLS using erythrocyte ghosts revealed pores not more than 45 Å in diameter even at high SLS concentrations, whereas those caused by SLO exceeded 128 Å in diameter (Buckingham and Duncan, 1983). Kinetic experiments further demonstrated that SLO lesions were formed within 1–2 minutes, whereas release of marker molecules from streptolysin S-treated vesicles began only after a 5–15 minute lag period (Buckingham and Duncan, 1983).

Recent studies by Carr *et al*., using a fixed concentration of SLS and varying numbers of target erythrocytes and vice versa, gave dose-dependent response curves consistent with a one-hit mechanism of hemolysis. This

mechanism is similar to complement-mediated lysis, where one molecule of SLS is sufficient to lyse one cell in a time- and temperature-dependent fashion (Carr *et al*., 2001). Further, SLS was shown to become an integral membrane protein and form pores with a predicted size between 0.14 and 0.36 nm, thereby leading to irreversible osmotic lysis of the cell (Carr *et al*., 2001).

## GENETIC BASIS OF SLS PRODUCTION

Prior to studies in our laboratory, and those in collaboration with Nizet *et al*. from the University of California, San Diego and Beall *et al*. from the Centers for Disease Control, nothing was known about the gene(s) encoding the hemolytic moiety of SLS in GAS. We initially demonstrated that a Tn*916* insertional inactivation of a promoter motif just upstream of a novel open reading frame (ORF), encoding a 53-amino acid peptide, abrogated β-hemolysis *in vitro* and reduced the virulence of an M1 GAS strain in a mouse model of tissue neurosis (Betschel *et al*., 1998; Borgia *et al*., 1997). This gene, named *sagA* for streptolysin-associate gene, was pivotal to the elucidation of the genetic control of SLS expression. Concomitantly, Beall *et al*. discovered loss of SLS activity via Tn*917* insertional inactivation of an ORF immediately downstream of *sagA* in an M49 strain. Shortly thereafter, a combined effort between all three groups led to the discovery of eight additional ORFs downstream of *sagA*, forming what is now known as the *sag* operon (Nizet *et al*., 2000). Targeted integrations in each of the nine genes (*sagA* to *sagI*) abrogated hemolytic activity, whereas integrations upstream or downstream of the operon did not, suggesting that each gene is required for SLS production. Unequivocal evidence was provided when the cloning of the entire nine-gene operon from the M49 strain into a non-hemolytic (SLS-deficient) strain of *Lactococcus lactis* conferred a stable hemolytic phenotype.

Upon closer examination of the locus at the amino acid level, it was apparent that the *sag* operon (Figure 42.1) was strikingly similar to bacteriocin operons from other Gram-positive as well as Gram-negative bacteria. Bacteriocins are small bacterial antimicrobial peptides that target specific bacteria other than the producing strain causing lysis. Synthesized first as a larger prepropeptide, which is comprised of a 23–26 amino acid N-terminal leader sequence and a 22–60 amino acid C-terminal propeptide, these antimicrobials are modified at specific residues (serine, threonine, glycine, and cysteine) and subsequently cleaved from their leader sequences before going on to produce a functional bacteriocin (Klaenhammer, 1993). *SagA*, thought to be the structural gene of SLS, encodes a 53-amino acid peptide (Figure 42.2) that is similar in size and amino acid composition to the prepropeptide of Microcin B17 from *Escherichia coli* (Yorgey *et al*., 1993). Also similar to many bacteriocins is the Gly-Gly proteolytic cleavage motif, which separates a putative 23 amino acid leader peptide from a 30 amino acid propeptide rich in the modifiable residues of Ser, Thr, Gly, and Cys (Figure 42.2). The *sag* locus (Figure 42.1) also contains candidate genes required for propeptide chemical modification (*sagB*), self-protection or immunity (*sagE*), an ABC transporter for export and maturation proteolysis of the leader peptide (*sagG* to *sagI*), and an internal terminator motif. The latter allows for differential transcription of structural gene and accessory gene mRNAs so as to allow for an abundance of structural gene (*sagA*) transcripts (Nizet *et al*., 2000).

Transcripts of the *sagA* gene are maximally produced at six hours following mid-exponential phase (Betschel *et al*., 1998) in keeping with maximal SLS production in the stationary phase of growth determined in early biochemical studies on SLS. Also, the predicted size of the SagA propeptide matches the size of mature SLS calculated from earlier biochemical analyses (Koyama and Egami, 1963; Lai *et al*., 1978; Loridan and Alouf, 1986).

| GAS | A | **SagB** (316 aa) | **SagC** (352 aa) | **SagD** (452 aa) | **SagE** (223 aa) | **SagF** (227 aa) | **SagG** (307 aa) | **SagH** (375 aa) | **SagI** (372 aa) |
|---|---|---|---|---|---|---|---|---|---|
| *S. iniae* | | 316 (77%) | 354 (78%) | 453 (82%) | 220 (60%) | 229 (52%) | 307 (75%) | 375 (81%) | 372 (74%) |
| GGS | | 316 (81%) | 182 (74%) | not sequenced | | | | | |
| | | Modification | Unknown | Unknown | Immunity | Unknown | ATP-binding cassette transporter | | |
| | | 36 kDa | 40 kDa | 52 kDa | 25 kDa | 26 kDa | 34 kDa | 42 kDa | 42 kDa |

**FIGURE 42.1** Schematic representation of sag operon from GAS with deduced amino acid identities among *sag* operons from Group G *Streptococcus* (GGS), and from *S. iniae* (SI). Also shown are the predicted sizes and putative functions of all 8 accessory gene products (SagB to SagI). Percent identities in parentheses reflect amino acid comparisons with GAS.

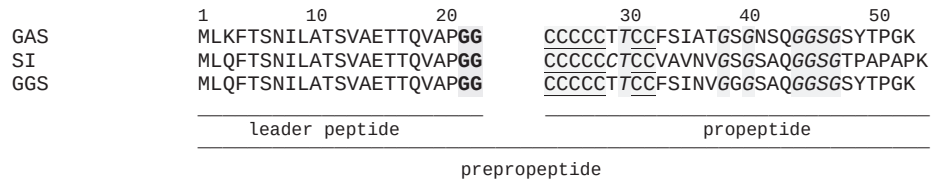



**FIGURE 42.2** Predicted amino acid homologies of SLS (SagA) from Groups A (GAS) and G (GGS), and from *S. iniae* (SI). Highlighted is the putative Gly-Gly (bold) cleavage site, as well as conserved residues hypothesized to undergo post-translational modification [Cys (underlined), Thr, Ser, and Gly (italic)].

Moreover, the previous unsuccessful attempts at sequencing the SLS peptide by Edman degradation may reflect cyclical thioether bridge structures or N-terminal blockage by a 2-oxobutyryl group as seen in other such molecules (Meyer *et al*., 1994). Since cysteine was not detected in purified SLS (Loridan and Alouf, 1986; Alouf and Loridan, 1988), the presence of seven cysteines, five adjacent to each other, is interesting and supports post-translational modification of these residues.

Processing of SagA and its export through the cell membrane are not yet understood. However, HtrA (DegP), a serine protease involved in the folding and maturation of secreted proteins, was shown to affect SLS production (Lyon and Caparon, 2004). An *htr*A insertional mutant showed high levels of SLS activity with expression occurring earlier in the growth phase than with the wild-type. The authors proposed that HtrA either may function to degrade components of the SLS biogenesis machinery or may even affect the tethering of SLS to the cell surface. Interestingly, the increased SLS activity in the mutant did not correlate with an increase in disease severity in a murine subcutaneous infection model; perhaps Htr also affects other virulence determinants that were not examined in this study.

## REGULATION

As described for many GAS determinants, the regulation of *sagA* and SLS activity is complex and has been shown to be influenced by a number of regulators. Furthermore, these regulators form an intricate network influenced by global regulators, making it difficult to determine which one directly regulates SLS expression. Results from studies examining *sagA* transcription do not always agree with those where SLS activity is used as a measure of transcript up-regulation. To complicate matters even further, the *sagA* transcript itself possesses the ability to transcriptionally and post-transcriptionally regulate the expression of other genes involved in the virulence of GAS. The regulation of SLS in GAS as determined by transcriptional analysis and by SLS activity is summarized in Figure 42.3.

### Regulation of SLS expression

Transcriptional regulators that have been shown to influence the expression of *sagA* include Mga, CovRS/CsrRS, the RofA-like family of regulators (RALPs), Rgg, and FasABC. Some of these regulators act directly on *sagA*, while others modulate regulatory networks affecting *sagA* expression. Mga, a global regulator that positively regulates a number of virulence genes involved in adhesion and invasion such as M protein, streptococcal inhibitor of complement, C5A-peptidase, and IgG-binding protein, negatively regulates SLS activity (Kreikemeyer *et al*., 2003) (Figure 42.3a and 42.3b). Thus, an Mga-deficient mutant was shown to exhibit increased cytoxicity to human lymphocytes due to increased SLS production relative to the wild-type (Kihlberg *et al*., 1999). Other negative regulators of *sagA* expression are RofA (Beckert *et al*., 2001) and Nra (Kreikemeyer *et al*., 2002), which belong to the RofA-like family of regulators (RALP) (Krekeimeyer, 2003). An Nra mutant was shown to escape from phagocytic vacuoles faster, due to increased expression of SLS and pyrogenic exotoxins A and B (SpeA and SpeB), suggesting that this regulator might be important in maintaining intracellular persistence of GAS *in vivo* (Molinari *et al*., 2001). Interestingly, Nra and RofA also negatively regulate Mga, but are themselves under positive regulation by Mga (Podbielski *et al*., 1999; Kreikemeyer *et al*., 2002). Consequently, it is difficult to determine which of these regulators might have a direct effect on *sagA* transcription, and mutations in any of them ultimately have an effect on SLS expression (Figures 42.3a and 42.3b).

The two-component signal transduction system CovRS (control of virulence regulator), also known as CsrRS (capsule synthesis regulator), also negatively regulates *sag*A (Figures 42.3a and 42.3b), as well as a number of other virulence determinants, including the *has* operon encoding hyaluronic acid capsule and

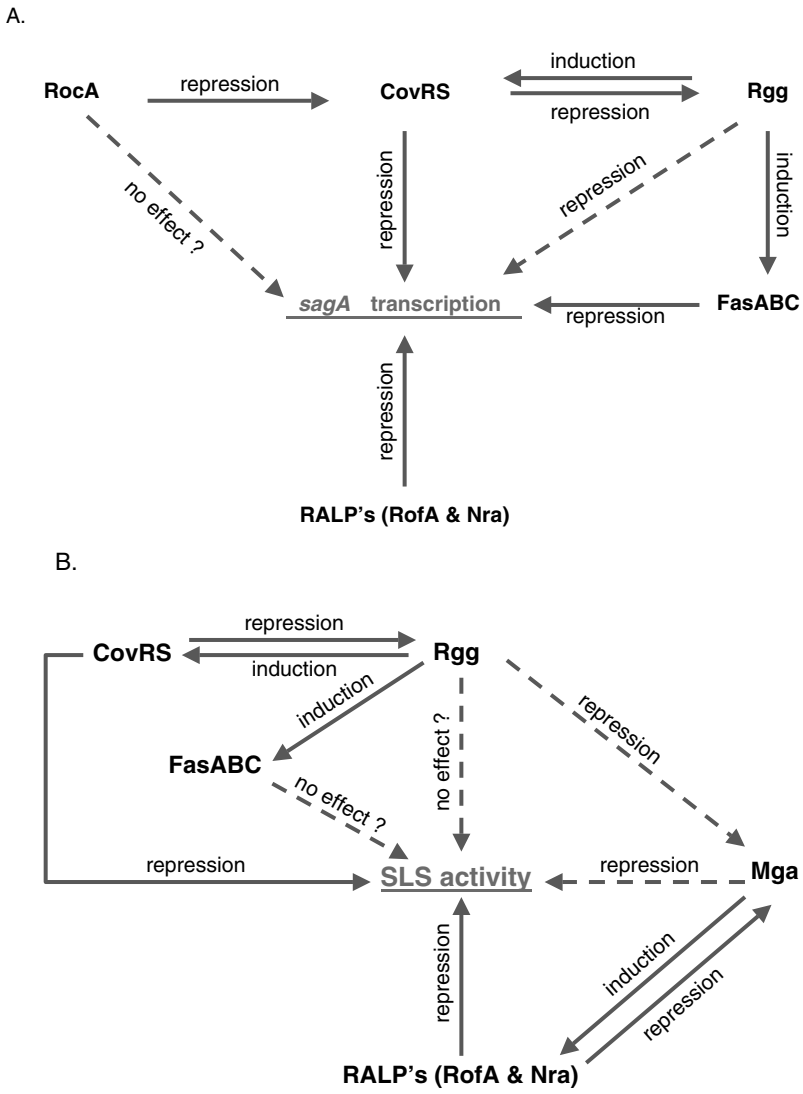



**FIGURE 42.3** Regulatory elements affecting *sagA* transcription (A) and SLS activity (B). Solid arrows indicate direct regulation. Dashed arrows indicate either no effect or inconclusive evidence as to direct or indirect regulation.

the global regulator Rgg/RopB (Federle *et al*., 1999; Graham *et al*., 2002; Heath *et al*., 1999; Levin and Wessels, 1998). CovRS was shown to directly bind the promoter region of the *sag* operon, indicating a direct influence on *sagA* transcription (Miller *et al*., 2001). DNA microarray studies demonstrated *sagA* expression to be increased almost eightfold in a CovRS-deficient mutant relative to the wild-type during the late exponential phase of growth (Graham *et al*., 2002). The transcription of the CovRS genes are themselves influenced by at least two regulators: RocA (regulator of CovR) acting as a negative regulator (Biswas and Scott, 2003) and Rgg (RopB) as a positive regulator (Chaussee *et al*., 2002) (Figure 42.3a and 42.3b). Interestingly, since RocA is a negative regulator of CovRS, which is in turn a negative regulator of *sagA*, one would expect increased expression of SLS in a RocA-deficient mutant, as was observed for hyaluronic acid synthesis (Biswas and Scott, 2003). However, this up-regulation was not observed for *sagA* and warrants further investigation. In addition to regulating CovRS, Rgg/RopB acts as a global transcriptional regulator of a number of genes associated with both metabolism and virulence, as well as the transcriptional regulators Mga and FasABC (Chaussee *et al*., 2001; Chaussee *et al*., 2002). An Rgg-deficient mutant was found to show up-regulation of *sagA* transcript expression (Chaussee *et al*., 2002). Although studies so far do not rule out the possibility that Rgg/RopB could be acting directly on the *sag* operon, the lack of conserved regions in the various promoters under Rgg/RopB control suggests it acts through modulation of other regulatory networks (Krekeimeyer, 2003).

Possible regulation of SagA at the post-transcriptional level comes from studies of the *fasABC* operon, which regulates *sagA* (Figure 42.3a), as well as genes encoding

fibronectin and fibrinogen-binding proteins, and streptokinase in a growth-phase dependent manner (Kreikemeyer *et al*., 2001; Kreikemeyer *et al*., 2003). In a *fasABC* knockout, the transcriptional profile of the aforementioned genes, except *sagA*, directly correlated with the functional activity of each gene product (Kreikemeyer *et al*., 2001). Although an increase in *sagA* mRNA was also observed, this did not correlate with a concomitant increase in SLS, suggesting that in addition to regulating *sagA* transcription, the *fas* operon might also have an effect on SagA transport and processing, which, as described previously, are likely complex.

### The *sagA* transcript as a regulator

Shortly after the identification of *sagA*, Li *et al*. demonstrated that a Tn*917* insertion in the promoter region of the *sagA* gene in an M49 serotype strain resulted in reduced expression of SLS as well as M protein, SpeB, and streptokinase (Li *et al*., 1999). Because of this pleiotropic effect, they designated it *pel* for pleiotropic effect locus. However, the regulatory effect of *sagA/pel* might be serotype dependent since Tn *916* insertions in the *sagA/pel* genes of an M1 and an M18 strain showed loss of SLS activity, but no effect on the production of cell-associated and extracellular proteins, capsule production, DNase, and caseinase activity (Betschel *et al*., 1998). In another study using an M6 serotype strain, the level of *emm* transcript encoding M protein remained unaltered in the *sagA/pel* insertion-deletion mutant, but the M protein was produced in a truncated form lacking its C-terminal domain and was not anchored to the cell surface (Biswas *et al*., 2001). The authors speculated that *sagA* or one of the other genes in the *sag* operon might directly or indirectly decrease the expression of a protease(s) that could truncate M protein. Interestingly, when the M49 *sagA/pel* Tn*917* mutant was passaged in mice, SpeB and streptokinase activity remained down-regulated, but both β-hemolysis and M protein expression were restored despite maintenance of Tn*917* in the *sagA/pel* promoter (Eberhard *et al*., 2001). The authors suggested that additional levels of regulation of SLS expression were selected for by biological pressures *in vivo*.

Further evidence to support *sagA* as a regulatory element comes from a recent study of an M1 serotype strain (Mangold *et al*., 2004). A *sagA*-deficient mutant of this strain not only showed loss of the β-hemolytic phenotype, but also reduced transcription of at least nine genes, including those for M-protein, streptokinase, streptococcal inhibitor of complement, pyrogenic exotoxin C, and NAD-glycohydrolase. Most interesting was the fact that although *speB* transcripts were produced normally, SpeB production was delayed and did not appear until late stationary phase in the mutant, compared with the appearance of activity in late logarithmic phase in the wild-type. Western blot analysis showed that although the mutant secreted the inactive zymogen of SpeB, processing to the active form was delayed. The authors concluded that the *sagA* transcript influenced processing and maturation of SpeB. Thus, in addition to acting as a transcriptional regulator, *sagA* was also acting as a regulator at the post-transcriptional level.

## ROLE OF SLS IN DISEASE PATHOGENESIS

The majority of clinical isolates of GAS, regardless of serotype, produce SLS. Naturally occurring strains of SLS-negative GAS are rare and although such clinical isolates occur, they often are not recognized because non-hemolytic mutants from throat swabs are dismissed as viridans streptococci in the clinical laboratory (Nida and Cleary, 1983). Consequently, it has not been possible to attribute a specific role to SLS based on SLS production by strains associated with a particular disease manifestation. Numerous studies in the past, using purified SLS preparations, attempted to examine the role of SLS in pathogenesis. However, the difficulty of obtaining purified SLS preparations made such studies problematic. Furthermore, the SLS peptide is non-immunogenic, and antibodies against purified SLS have neither been experimentally generated nor detected in sera from patients with GAS infections.

Although the cytotoxic effect of SLS *in vitro* has been demonstrated on a wide variety of cells, what consequence this might have *in vivo* is debatable. Pathological effects of SLS *in vivo* have been described. Mice and rabbits injected intravenously with SLS showed degenerative changes in kidney tubules and glomerulitis, respectively, whereas intramuscular injections showed muscle degeneration and sub-acute inflammation (Ginsburg, 1970). Intravenous injection of large doses of SLS in rabbits caused massive intravascular hemolysis followed by death (Ginsburg, 1970). By contrast, SLS specifically injected into the knee joints of rabbits produced acute arthritis followed by a chronic form on repeated injection (Weissmann *et al*., 1965). However, the purity of SLS may have been questionable in these studies, and these observations could have resulted from contaminants (Ginsburg, 1970).

It has only been through more recent work using SLS-deficient mutants that insight has been gained on the possible role of SLS in pathogenesis. It was not until

1998 that studies in our laboratory provided evidence implicating SLS as a virulence factor associated with skin infection. We showed that an SLS-deficient mutant with a *Tn916* insertion in the *sagA* locus of an M1 strain caused significantly fewer and smaller necrotic lesions and had no adverse effect on mouse weight gain when injected subcutaneously at $10^6$ CFU, compared with the wild-type, which caused weight loss and a necrotic lesion within 72 hours (Betschel *et al*., 1998). Histological examination of the lesion revealed intense neutrophil infiltration, as well as tissue necrosis with the wild-type but not with SLS-deficient mutants. Further work using targeted knockouts in each of the genes of the *sag* locus confirmed that these effects were due to SLS (Nizet *et al*., 2000).

More recently, using double mutants of an M1 strain that were SLS-deficient (Δ*sag*A) and capsule positive (Δ*cov*RS), SLS was shown to correlate with lesion size and together with capsule to be responsible for subcutaneous spread of infection and mortality in the murine model (Engleberg *et al*., 2004). Another study using SLO- and SLS (Δ*sagB*)-deficient mutants of an M5 strain also demonstrated that SLS contributes to lesion size in mice (Fontaine *et al*., 2003). Interestingly, a double-mutant deficient in both SLS as well as SLO produced lesions in fewer mice at $10^6$ CFU than either the SLS- or SLO-deficient mutants alone, indicating that SLO also contributes to necrosis. In this study, $LD_{50}$ was shown to be decreased by 1 log with an SLS-deficient mutant compared with the parent when injected subcutaneously, but no difference was observed with intraperitoneal injection (Fontaine *et al*., 2003), indicating that SLS affects mortality depending on the route of infection. Moreover, mortality may be a function of M type, since in a study utilizing an M3 strain neither SLS- nor SLO-deficient mutants had an effect on mouse survival when injected subcutaneously, and SLS was found to have a slightly lesser contribution to mortality than SLO when mutants were injected intraperitoneally in a murine sepsis model (Sierig *et al*., 2003).

Purified SLS has also been shown to inhibit phagocytosis *in vitro* (Ofek *et al*., 1972). Recently, Dale *et al*. generated antibodies in rabbits, using a synthetic peptide based on amino acid residues 10–30 of the SagA propeptide, which neutralized the activity of SLS *in vitro* and which significantly enhanced phagocytosis when combined with type-specific M protein antibodies (Dale *et al*., 2002). However, *in vitro* studies using M3 SLS- and SLO-deficient mutants showed that SLO but not SLS was associated with impaired polymorphonuclear leukocyte killing and only in an acapsular background (Sierig *et al*., 2003). Thus, the inhibition of phagocytosis seen with purified SLS (Ofek *et al*., 1972), which is obviously of advantage in combating the host immune response, does not appear to occur in the presence of other GAS virulence determinants *in vitro*. However, the intense neutrophil recruitment, which occurs at the site of GAS infection in the murine subcutaneous model without concomitant bacterial clearance, and which is not seen with SLS-deficient mutants, suggests that SLS *in vivo* may affect neutrophil function (Betschel *et al*., 1998). Further studies are needed to determine the synergistic effects occurring with SLS and other GAS virulence determinants *in vivo*.

## SLS PRODUCTION IN OTHER PATHOGENIC STREPTOCOCCI

Following the identification of the *sag* operon in GAS, *sag* operon homologues have been identified in other streptococci. A *sag* operon encoding SLS was identified in GGS strains isolated from patients with necrotizing soft tissue infections (Humar *et al*., 2002). Similar to GAS, a GGS mutant with an inactivation of the *sagA* gene was incapable of forming necrotic lesions in mice as demonstrated by the parent strain (Humar *et al*., 2002). A GCS strain was also demonstrated to produce SLS (Flanagan *et al*., 1998) and to possess *sagA* (Steiner and Malke, 2002). In the latter study, homologues of *cov*RS and the *fas* operon were identified in GCS, and as found in GAS, act as positive and negative regulators of SLS, respectively (Steiner and Malke, 2002). Similarly, the hemolytic phenotype of *S. iniae*, a fish pathogen that also causes soft tissue infections in humans (Weinstein *et al*., 1997), was shown to be due to SLS production, and *sag*-deficient mutants showed SLS to be responsible for necrotic lesion formation in a murine model of subcutaneous infection (Fuller *et al*., 2002). Amino acid sequence comparisons of *sag* homologues reveal striking similarities with *sag*A from GAS, in particular those regions common to bacteriocins, as well as with other *sag* genes (Figure 42.1 and 42.2).

## CONCLUSION

Great strides in the understanding of SLS expression and its contribution to disease have been made in the last decade. Evidence for the dual role of *sagA*, both as the structural gene for SLS as well as a regulator, has emerged. However, many questions remain to be answered. The amino acid structure of the SLS peptide has not been elucidated, nor have the theoretical predictions regarding its processing been verified. Whether the *sagA* transcript alone acts as a regulator or

whether SLS may also function as a quorum-sensing molecule deserves further investigation. The sometimes conflicting results, both in terms of the regulatory capacity of the *sagA* transcript and the role in pathogenesis of *sag* knockout mutants of different M types, suggest that the role of the *sag* operon might differ in different backgrounds. The importance of strain background is not to be underestimated in experiments employing knockout mutants to assess virulence, since genome sequences of GAS strains of differing serotypes show considerable differences. Not only are the regulatory pathways in GAS complex with possible differences in different M types, but regulation of expression of virulence determinants in different biological niches likely varies. For example, we have observed that a capsule negative M1 strain remains acapsular in a mouse skin lesion, but expresses capsule when in the bloodstream (S. Betschel, unpublished data). Although the studies done so far using murine models of subcutaneous infection confirm the contribution of SLS to lesion formation, not all serotypes may show the same outcome. The use of a variety of infection models, as well as mutants of different backgrounds, should help to establish the precise role of SLS in disease. Given the progress made in the research on GAS virulence determinants in recent years, it is our hope that the next decade will shed further light on SLS, which still remains a fascinating and elusive peptide almost 70 years after its discovery.

## REFERENCES


Akao, T., Hashimoto, S., Kobashi, K. and Hidaka, Y. (1999). Unique synthetic peptides stimulating streptolysin S production in streptococci. *J.Biochem.(Tokyo.)* **125,** 27–30.

Akao, T., Takahashi, T. and Kobashi, K. (1992). Purification and characterization of a peptide essential for formation of streptolysin S by *Streptococcus pyogenes*. *Infect.Immun*. **60,** 4777–4780.

Alouf, J.E. (1980). Streptococcal toxins(streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol.Ther*. **11,** 661–717.

Alouf, J.E. and Loridan, C. (1988). Production, purification, and assay of streptolysin S. *Methods Enzymol*. **165,** 59–64.

Beckert, S., Kreikemeyer, B. and Podbielski, A. (2001). Group A streptococcal *rofA* gene is involved in the control of several virulence genes and eukaryotic cell attachment and internalization. *Infect.Immun*. **69,** 534–537.

Betschel, S.D., Borgia, S.M., Barg, N.L., Low, D.E. and De Azavedo, J.C. (1998). Reduced virulence of group A streptococcal *Tn916* mutants that do not produce streptolysin S. *Infect.Immun*. **66,** 1671–1679.

Biswas, I., Germon, P., McDade, K. and Scott, J.R. (2001). Generation and surface localization of intact M protein in *Streptococcus pyogenes* are dependent on sagA. *Infect.Immun*. **69,** 7029–7038.

Biswas, I. and Scott, J.R. (2003). Identification of *rocA*, a positive regulator of covR expression in the group A Streptococcus. *J.Bacteriol*. **185,** 3081–3090.

Borgia, S.M., Betschel, S., Low, D.E. and De Azavedo, J.C. (1997). Cloning of a chromosomal region responsible for streptolysin S production in *Streptococcus pyogenes*. *Adv.Exp.Med.Biol*. **418,** 733–736.

Buckingham, L. and Duncan, J.L. (1983). Approximate dimensions of membrane lesions produced by streptolysin S and streptolysin O. *Biochim.Biophys.Acta* **729,** 115–122.

Calandra, G.B. and Cole, R.M. (1981). Membrane and cytoplasmic location of streptolysin S precursor. *Infect.Immun*. **31,** 386–390.

Carr, A., Sledjeski, D.D., Podbielski, A., Boyle, M.D. and Kreikemeyer, B. (2001). Similarities between complement-mediated and streptolysin S-mediated hemolysis. *J.Biol.Chem*. **276,** 41790–41796.

Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Smoot, L.M., Graham, M.R., Watson, R.O. and Musser, J.M. (2002). Rgg influences the expression of multiple regulatory loci to co-regulate virulence factor expression in *Streptococcus pyogenes*. *Infect.Immun*. **70,** 762–770.

Chaussee, M.S., Watson, R.O., Smoot, J.C. and Musser, J.M. (2001). Identification of Rgg-regulated exoproteins of *Streptococcus pyogenes*. *Infect.Immun*. **69,** 822–831.

Dale, J.B., Chiang, E.Y., Hasty, D.L. and Courtney, H.S. (2002). Antibodies against a synthetic peptide of SagA neutralize the cytolytic activity of streptolysin S from group A streptococci. *Infect.Immun*. **70,** 2166–2170.

Eberhard, T.H., Sledjeski, D.D. and Boyle, M.D. (2001). Mouse skin passage of a *Streptococcus pyogenes* Tn917 mutant of sagA/pel restores virulence, beta-hemolysis, and sagA/pel expression without altering the position or sequence of the transposon. *BMC. Microbiol*. **1,** 33.

Elias, N., Heller, M. and Ginsburg, I. (1966). Binding of streptolysin S to red blood cell ghosts and ghost lipids. *Isr. J. Med. Sci*. **2,** 302–309.

Engleberg, N.C., Heath, A., Vardaman, K. and DiRita, V.J. (2004). Contribution of CsrR-regulated virulence factors to the progress and outcome of murine skin infections by *Streptococcus pyogenes*. *Infect.Immun*. **72,** 623–628.

Federle, M.J., McIver, K.S. and Scott, J.R. (1999). A response regulator that represses transcription of several virulence operons in the group A streptococcus. *J. Bacteriol*. **181,** 3649–3657.

Flanagan, J., Collin, N., Timoney, J., Mitchell, T., Mumford, J.A. and Chanter, N. (1998). Characterization of the hemolytic activity of Streptococcus equi. *Microb. Pathog*. **24,** 211–221.

Fontaine, M.C., Lee, J.J. and Kehoe, M.A. (2003). Combined contributions of streptolysin O and streptolysin S to virulence of serotype M5 *Streptococcus pyogenes* strain *Manfredo*. *Infect. Immun*. **71,** 3857–3865.

Fuller, J.D., Camus, A.C., Duncan, C.L., Nizet, V., Bast, D.J., Thune, R.L., Low, D.E. and De Azavedo, J.C. (2002). Identification of a streptolysin S-associated gene cluster and its role in the pathogenesis of *Streptococcus iniae* disease. *Infect. Immun*. **70,** 5730–5739.

Ginsburg, I. (1970) In: *Microbial Toxins Streptolysin S, Vol III*, (ed. T.C. Montie, S. Kadis and S.J. Ajl), pp. 99–171. Academic Press, New York, NY.

Ginsburg, I. (1999). Is streptolysin S of group A streptococci a virulence factor? *APMIS* **107,** 1051–1059.

Ginsburg, I., Harris, T.N. and Grossowicz, N. (1963). Oxygen-stable hemolysins of group A Streptococci. I. The role of various agents in the production of the hemolysins. *J. Exp. Med*. **118,** 905–917.

Graham, M.R., Smoot, L.M., Migliaccio, C.A., Virtaneva, K., Sturdevant, D.E., Porcella, S.F., Federle, M.J., Adams, G.J., Scott, J.R. and Musser, J.M. (2002). Virulence control in group A streptococcus by a two-component gene regulatory system: global expression profiling and *in vivo* infection modeling. *Proc. Natl. Acad. Sci. USA* **99,** 13855–13860.

Heath, A., DiRita, V.J., Barg, N.L. and Engleberg, N.C. (1999). A two-component regulatory system, CsrR-CsrS, represses expression

of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. *Infect. Immun*. **67,** 5298–5305.

Humar, D., Datta, V., Bast, D.J., Beall, B., De Azavedo, J.C. and Nizet, V. (2002). Streptolysin S and necrotizing infections produced by group G streptococcus. *Lancet* **359,** 124–129.

Kihlberg, B.M., Collin, M., Olsen, A. and Bjorck, L. (1999). Protein H, an antiphagocytic surface protein in *Streptococcus pyogenes*. *Infect. Immun*. **67,** 1708–1714.

Klaenhammer, T.R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*. **12,** 39–85.

Koyama, J. and Egami, F. (1963). Biochemical studies on streptolysin S′ formed in the presence of yeast ribonucleic acid. I. The purification and some properties of the toxin. *J. Biochem*. (Tokyo) **53,** 147–154.

Kreikemeyer, B., Beckert, S., Braun-Kiewnick, A. and Podbielski, A. (2002). Group A streptococcal RofA-type global regulators exhibit a strain-specific genomic presence and regulation pattern. *Microbiology* **148,** 1501–1511.

Kreikemeyer, B., Boyle, M.D., Buttaro, B.A., Heinemann, M. and Podbielski, A. (2001). Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas) with homologies to two-component type regulators requires a small RNA molecule. *Mol. Microbiol*. **39,** 392–406.

Kreikemeyer, B., McIver, K.S. and Podbielski, A. (2003). Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. *Trends Microbiol*. **11,** 224–232.

Lai, C.Y., Wang, M.T., de Faria, J.B. and Akao, T. (1978). Streptolysin S: improved purification and characterization. *Arch. Biochem. Biophys*. **191,** 804–812.

Levin, J.C. and Wessels, M.R. (1998). Identification of csrR/csrS, a genetic locus that regulates hyaluronic acid capsule synthesis in group A streptococcus. *Mol. Microbiol*. **30,** 209–219.

Li, Z., Sledjeski, D.D., Kreikemeyer, B., Podbielski, A. and Boyle, M.D. (1999). Identification of pel, a Streptococcus pyogenes locus that affects both surface and secreted proteins. *J. Bacteriol*. **181,** 6019–6027.

Loridan, C. and Alouf, J.E. (1986). Purification of RNA-core induced streptolysin S, and isolation and hemolytic characteristics of the carrier-free toxin. *J. Gen. Microbiol*. **132,** 307–315.

Lyon, W.R. and Caparon, M.G. (2004). Role for serine protease HtrA (DegP) of *Streptococcus pyogenes* in the biogenesis of virulence factors SpeB and the hemolysin streptolysin S. *Infect. Immun*. **72,** 1618–1625.

Mangold, M., Siller, M., Roppenser, B., Vlaminckx, B.J., Penfound, T.A., Klein, R., Novak, R., Novick, R.P. and Charpentier, E. (2004). Synthesis of group A streptococcal virulence factors is controlled by a regulatory RNA molecule. *Mol. Microbiol*. **53,** 1515–1527.

Meyer, H.E., Heber, M., Eisermann, B., Korte, H., Metzger, J.W. and Jung, G. (1994). Sequence analysis of lantibiotics: chemical derivatization procedures allow a fast access to complete Edman degradation. *Anal. Biochem*. **223,** 185–190.

Miller, A.A., Engleberg, N.C. and DiRita, V.J. (2001). Repression of virulence genes by phosphorylation-dependent oligomerization of CsrR at target promoters in *S. pyogenes*. Mol. Microbiol. ***40,*** 976–990.

Molinari, G., Rohde, M., Talay, S.R., Chhatwal, G.S., Beckert, S. and Podbielski, A. (2001). The role played by the group A streptococcal negative regulator Nra on bacterial interactions with epithelial cells. *Mol. Microbiol*. **40,** 99–114.

Nida, K. and Cleary, P.P. (1983). Insertional inactivation of streptolysin S expression in *Streptococcus pyogenes*. *J. Bacteriol*. **155,** 1156–1161.

Nizet, V. (2002). Streptococcal beta-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol*. **10,** 575–580.

Nizet, V., Beall, B., Bast, D.J., Datta, V., Kilburn, L., Low, D.E. and De Azavedo, J.C. (2000). Genetic locus for streptolysin S production by group A streptococcus. *Infect. Immun*. **68,** 4245–4254.

Ofek, I., Bergner-Rabinowitz, S. and Ginsburg, I. (1972). Oxygen-stable hemolysins of group A streptococci. 8. Leukotoxic and antiphagocytic effects of streptolysins S and O. *Infect. Immun*. **6,** 459–464.

Palmer, M., Vulicevic, I., Saweljew, P., Valeva, A., Kehoe, M. and Bhakdi, S. (1998). Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* **37,** 2378–2383.

Podbielski, A., Woischnik, M., Leonard, B.A. and Schmidt, K.H. (1999). Characterization of nra, a global negative regulator gene in group A streptococci. *Mol. Microbiol*. **31,** 1051–1064.

Sierig, G., Cywes, C., Wessels, M.R. and Ashbaugh, C.D. (2003). Cytotoxic effects of streptolysin o and streptolysin s enhance the virulence of poorly encapsulated group A streptococci. *Infect. Immun*. **71,** 446–455.

Steiner, K. and Malke, H. (2002). Dual control of streptokinase and streptolysin S production by the covRS and fasCAX two-component regulators in *Streptococcus dysgalactiae* subsp. equisimilis. *Infect. Immun*. **70,** 3627–3636.

Taketo, A. and Taketo, Y. (1964). Biochemical studies on streptolysin S formation. I. Streptolysin S formation in cell free system. *J. Biochem.(Tokyo)*, **56,** 552–561.

Thelestam, M. and Mollby, R. (1979). Classification of microbial, plant, and animal cytolysins based on their membrane-damaging effects of human fibroblasts. *Biochim. Biophys. Acta* **557,** 156–169.

Theodore, T.S. and Calandra, G.B. (1981). Streptolysin S activation by lipoteichoic acid. *Infect. Immun*. **33,** 326–328.

Todd, E.W. (1938). The differentiation of two distinct serological varieties of streptolysins: streptolysin O and streptoilysin S. *J. Pathol. Bacteriol* **47,** 423–445.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B.M., Borczyk, A. and Low, D.E. (1997). Invasive infections due to a fish pathogen, *Streptococcus iniae*. *N. Engl. J. Med*. **337,** 589–594.

Weissmann, G., Becher, B., Wiedermann, G. and Bernheimer, A.W. (1965). Studies on lysosomes. Vii. Acute and chronic arthritis produced by intra-articular injections of streptolysin S in rabbits. *Am. J. Pathol*. **46,** 129–147.

Weld, J. (1934). The toxic properties of serum extracts of hemolytic streptococci. *J. Exp. Med*. **59,** 83–95.

Yorgey, P., Davagnino, J. and Kolter, R. (1993). The maturation pathway of microcin B17, a peptide inhibitor of DNA gyrase. *Mol. Microbiol*. **9,** 897–905.

CHAPTER

# 43

# The group B streptococcal β-hemolysin/cytolysin

*George Y. Liu and Victor Nizet*

## INTRODUCTION

Group B *Streptococcus* (GBS) is the leading cause of invasive bacterial infections in human newborns and is increasingly recognized as a pathogen in adult populations, including the elderly, pregnant women, and diabetics (Berner, 2004; Blancas *et al*., 2004). The GBS β-hemolysin/cytolysin (βH/C) is a surface-associated toxin that plays an important role in virulence of the organism. The βH/C is also responsible for the characteristic zone of clearing around GBS colonies grown on blood agar plates, a useful diagnostic phenotype in the clinical microbiology laboratory.

The GBS βH/C was recognized as early as the 1930s (Todd, 1934), but was largely neglected from an experimental standpoint. Nearly a half-century later, the emergence of GBS invasive disease in newborns triggered renewed interest in the toxin as a potential virulence factor of the pathogen. Studies in the early 1980s provided insight on the basic biochemical properties of the βH/C and its mechanism of erythrocyte lysis. However, further in-depth analysis was hindered by the toxin's lability and apparent non-immunogenicity, factors that to this day pose great challenges to final purification. In the 1990s, the advent of molecular genetic tools for GBS analysis allowed generations of isogenic mutant strains with altered βH/C phenotypes. With this technological breakthrough, the role of βH/C in GBS disease has been explored extensively in tissue culture and small animal models of infection. These studies have shown a toxin with a broad array of cytolytic, proinvasive, proapoptotic, and proinflammatory properties that contribute in a significant fashion to the virulence of GBS (Nizet, 2002; Liu *et al*., 2004). Current knowledge thus suggests the βH/C may represent an attractive therapeutic target in management of neonatal GBS disease.

## BIOCHEMISTRY AND GENETICS

### Basic properties of βH/C

The first report of the GBS βH/C provided by Todd (1934) described an extracellular molecule that is oxygen stable, acid and heat labile, and non-immunogenic. Analogies were drawn to streptolysin S (SLS) of group A *Streptococcus* (GAS), because the two β-hemolysins share many properties at first glance, including their inherent lability, broad-spectrum cytolytic activity, and lack of immunogenicity (Ginsburg, 1970). However, genetic evidence to be discussed below has ultimately revealed these toxins to be unrelated molecules (Nizet, 2002).

Biophysical properties of the GBS βH/C have placed technical hurdles in the path of its purification. The molecule is acid and heat labile, with significant loss of activity at 50°C after 15 minutes (Dal and Monteil, 1983). Normally associated with the bacterial cell surface, GBS βH/C activity is facilitated through direct contact between the bacterium and the target eukaryotic cell (Platt, 1995). Simple culture supernatants of GBS do not possess measurable βH/C activity. However, the presence of certain high-molecular weight "carrier" molecules in solution can stabilize extracellular toxin activity (Ginsburg, 1970).

As experimental proof, placement of an 0.22 micron filter between the GBS and the target erythrocyte blocks hemolysis unless a carrier molecule is added to the medium (Platt, 1995). Examples of carrier molecules that have shown an ability to stabilize βH/C activity include serum proteins such as albumin, Tween 80 and related detergents, starch polymers, and lipotechoic acid (Marchlewicz and Duncan, 1980; Ferrieri, 1982; Tsaihong and Wennerstrom, 1983). In attempted protocols for βH/C purification, separation of the candidate toxin from its carrier molecule has invariably been associated with loss of hemolytic activity.

In an early report on partial purification of the GBS βH/C activity (Marchlewicz and Duncan, 1980), researchers harvested GBS in late log growth phase, resuspended the bacteria in phosphate buffered saline supplemented with 1% glucose plus the stabilizers Tween 80 and starch, and then concentrated the supernatant by methanol or ammonium chloride precipitation. When the extract was applied to a Sephacryl S-300 column, two distinct peaks were noted, one of which harbored most of the hemolytic activity. However, electrophoretic separation of this peak revealed the presence of numerous protein bands. In a subsequent published protocol, βH/C was extracted from a GBS pellet by sonication in a buffer containing bovine serum albumin (BSA) and further purified by gel filtration (Dal and Monteil, 1983). Eluent from the column also produced two peaks, the first of which on electrophoresis resolved into four protein bands. The top three bands were nonhemolytic and consisted of BSA polymer, dimer, and monomer. The front running band was hemolytic, but harbored traces of BSA. The authors attempted further purification by agarose gel electrophoresis or affinity chromatography on protein-absorbing Blue Sepharose CL6B, but these techniques failed to separate hemolytic activity from the BSA carrier.

The above observation of a hemolytic front running band smaller than the size of BSA monomer (about 67 kDa) appears to place an upper limit on the size of the mature toxin (Dal and Monteil, 1983). Moreover, during efforts to purify the βH/C following extraction of GBS with detergent or lipoteichoic acid (LTA), it was observed that βH/C can be bound to these macromolecules without significantly affecting their respective elution profiles macromolecule on column chromatography, suggesting that the βH/C is likely to be a relatively small molecule (Tsaihong and Wennerstrom, 1983).

GBS βH/C expression occurs primarily between the early exponential and early stationary phases of growth (Platt, 1995). In a GBS chemostat system where βH/C is constantly extracted with Tween 40 in the growth medium, optimal release was measured during the late exponential phase. βH/C production also increases significantly under experimental conditions of enhanced GBS growth rate (Ross *et al*., 1999). Production of the toxin is greatly enhanced if a low concentration of glucose (about 2 mg/ml) or other fermentable carbohydrates is present in the medium, but unexpectedly, glucose in the range of 10 mg/ml almost entirely abolished βH/C production, pointing to regulatory linkages of βH/C production to cellular metabolism (Tapsall, 1986). Consistent with this hypothesis, treatment with various metabolic inhibitors including sodium fluoride, iodoacetate, or 2-deoxy-glucose greatly reduced the production of assayable βH/C.

## Mechanism of βH/C pore formation

It is notable that GBS βH/C is cytolytic for a very broad range of eukaryotic cells, including erythrocytes, fibroblasts (Tapsall and Phillips, 1991), lung epithelial and endothelial cells (Nizet *et al*., 1996; Gibson *et al*., 1999), brain endothelial cells (Nizet *et al*., 1997b), and macrophages and neutrophils (Liu *et al*., 2004). By implication, the βH/C must interact with and damage molecular targets common to the cell membranes of all these cells. Though these host molecules have not been fully defined, it is notable that βH/C activity can be markedly inhibited by phospholipids, such as dipalmitolyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylethanolamine (DPPE) (Tapsall and Phillips, 1991). This finding suggests that the GBS βH/C may possess an affinity for similar phospholipids in the eukaryotic cell membrane bilayer and that this affinity could guide the toxin to its site of action. In contrast with streptolysin O (SLO) of GAS and related cytolytic toxins, cholesterol does not inhibit GBS βH/C activity.

Membrane pore formation is thought to represent a principal mechanism by which the GBS βH/C exerts its toxic activity, and this phenomenon has been most closely examined in studies of erythrocyte lysis. Marchlewicz and Duncan showed that upon incubation of GBS βH/C extract with a 1% suspension of erythrocytes at 37˚C, sufficient toxin associated with the target cells to produce 80% lysis within three minutes (Marchlewicz and Duncan, 1981). βH/C binding to erythrocytes appeared irreversible, unlike the interactions observed with SLS of GAS (Duncan and Mason, 1976). Also in marked contrast to SLS action, GBS βH/C exhibits a relatively short prelytic lag period, instead resembling lysis by GAS SLO or *Clostridium perfringens* theta-toxin (Bernheimer, 1947). Kinetic analysis revealed that rubidium ion and hemoglobin are released at the same rate from erythrocytes treated

with GBS βH/C, implying that the toxin creates membrane pores of relatively large size (Marchlewicz and Duncan, 1981).

Binding to erythrocytes by the βH/C, the length of the prelytic lag period, and the rate of hemolysis were found to be temperature dependent (Marchlewicz and Duncan, 1981). Since no toxin binding was noted at 0°C, it was proposed that a temperature-dependent fluidity of the membrane is necessary for GBS βH/C adsorption to the erythrocytes. As the incubation temperature increased, the length of the prelytic period decreased and the rate of lysis increased. Using an assay in which a fixed concentration of βH/C is incubated with an increasing concentrations of erythrocytes, the authors determined that more than one βH/C molecule is likely required to lyse a single cell (Marchlewicz and Duncan, 1981). When the logarithm of the fraction of unlysed RBC was plotted against βH/C concentration, a multi-hit survival curve was obtained (Atwood and Norman, 1949). Prediction based on this graph suggests that five molecules of βH/C are required to lyse a single sheep erythrocyte, reflecting a potency identical to that predicted for SLO of GAS (Inoue *et al*., 1976).

## Genetic basis of βH/C production

The first major advance toward understanding the genetics underlying GBS βH/C activity was achieved by Spellerberg and coworkers through negative selection (Spellerberg *et al*., 1999). Using a novel pGh9:ISS1 transposition vector, the group generated a mutant library from which they identifed several nonhemolytic GBS mutants. The transposon insertion sites all clustered within a seven kilobase genetic locus of the GBS chromosome that was named *cyl*, in reference to the cytolytic activity exhibited by the βH/C (Figure 43.1). This study and subsequent reports of the GBS *cyl* gene cluster identified 12 separate open reading frames (ORFs) organized in an operon structure (Spellerberg *et al*., 1999; Spellerberg *et al*., 2000; Pritzlaff *et al*., 2001). The deduced amino acid sequences of five of the genes (*cylD*, *cylG*, *acpC*, *cylZ, cylI*) demonstrate significant homologies to enzymes of prokaryotic fatty acid biosynthesis. Furthermore, the clustered arrangement of these genes in proximity to an acyl carrier protein (*acpC*) gene is reminiscent of the *fab* gene clusters of *Escherichiae coli, Vibrio harveyi*, and *Bacillus subtilis* (Rawlings and Cronan, 1992; Morbidoni *et al*., 1996; Shen and Byers, 1996). The predicted products of *cylA/B* share significant homologies to prokaryotic and eukaryotic ATP binding cassette transporters, which are known to be required for the hemolysin export in *E. coli* (Felmlee *et al*., 1985), *Bordetella pertussis* (Glaser *et al*., 1988), and *E. faecalis* (Gilmore *et al*., 1990, Chapter 41, this volume).

The association of the *cyl* locus with GBS βH/C production was corroborated through independent positive screening of a plasmid library of GBS chromosomal DNA in *E. coli* with the aim of identifying hemolytic transformants (Pritzlaff *et al*., 2001). One such hemolytic clone was isolated and the purified plasmid found to contain the GBS *cyl*E and downstream *cyl*F ORFs, as well as parts of the adjacent ORFs *cyl*B and *cyl*I. Confirmed targeted plasmid integrational disruption of *cyl*B, *cyl*F, and *cyl*I in three different GBS strains failed to consistently abrogate βH/C production. In contrast, targeted disruption of *cyl*E invariably produced a non-hemolytic phenotype, suggesting that of these genes, only *cyl*E was essential for βH/C expression (Pritzlaff *et al*., 2001). In each of the three strains in-frame allelic replacement of *cyl*E with an antibiotic cassette yields a NH (non-hemolytic) mutant

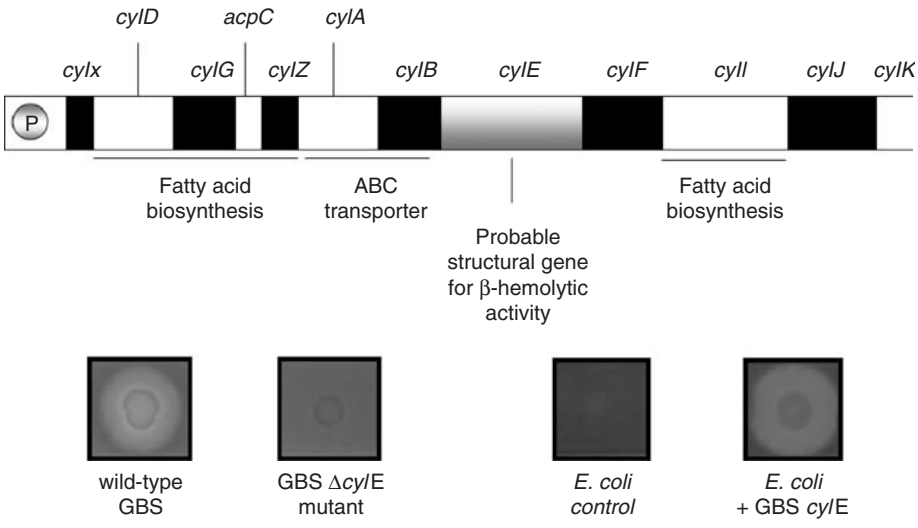



**FIGURE 43.1** Map of the GBS *cyl* operon encoding β-hemolysin/cytolysin activity. Allelic replacement of the *cyl*E ORF yields a nonhemolytic mutant, while heterologous expression of *cyl*E in *E. coli* is sufficient to confer a β-hemolytic phenotype (Spellerberg *et al*. 1999; Pritzlaff *et al*. 2002).

(Figure 43.1) in which βH/C activity can be restored by reintroduction of *cyl*E on a plasmid vector. When expressed in *E. coli* as a recombinant fusion protein, *cyl*E alone is sufficient to confer a robust β-hemolytic phenotype after 48 hours incubation (Pritzlaff *et al*., 2001) (Figure 43.1). The combination of the mutagenesis, complementation, and heterologous expression studies strongly suggests that *cyl*E encodes the structural determinant of the GBS βH/C, but does not exclude the possibility that additional GBS genes are involved in its processing, activation, or export to the cell surface. The predicted product of the *cyl*E ORF is a 78 kilodalton protein without significant homology to any other proteins in the GenBank databases. It remains to be determined whether the full-length protein or a derivative represents the mature toxin, and the lack of sequence homologies precludes further inference on the precise mechanism of βH/C action at this time.

Interestingly, GBS exhibiting a hyperhemolytic (HH) phenotype can be generated by chemical or transposon mutagenesis (Weiser and Rubens, 1987; Nizet *et al*., 1996). These observations suggest the existence of regulatory pathways that may contribute to the observed variations in βH/C expression with growth phase or glucose concentration, for example. Recently, one clear negative regulator of GBS *cyl* operon expression was identified, a homologue of the *cov*S-*cov*R two-component global transcriptional regulator that is so well studied in GAS (Lamy *et al*., 2004). A GBS *cov*S/R knockout mutant showed markedly increased βH/C activity, and *cov*R was shown to bind directly to the promoter motif at the head of the *cyl* operon (Lamy *et al*., 2004).

## Association of βH/C with pigment

An interesting feature of βH/C observed during biochemical and genetic studies is the close association of the βH/C to an orange pigment, also expressed on the surface of GBS. This pigment exhibits a triple peak absorbance at 455, 485, and 520 nm highly characteristic of β-carotene and related pigments (Merritt and Jacobs, 1978; Tapsall, 1986). The GBS carotenoid is quite labile and degrades readily to a single 425 nm peak when heated or subjected to freeze thawing. A close association with the βH/C was apparent because the pigment co-precipitated and co-purified with hemolytic activity (Tapsall, 1986). In fact, the inability to separate the two phenotypes led early investigators to conclude that the pigment could represent the natural carrier for the βH/C or even that the two molecules could be one and the same. However, published reports have since shown that the GBS βH/C and pigment are likely to be different molecules. For example, βH/C activity can be inactivated by subtilin, a serine protease. Other studies have shown that propagation of GBS under different pH and glucose concentrations results in differential yields of βH/C protein and pigment (Tapsall, 1987).

The close linkage between the GBS βH/C and carotenoid pigment holds true at the genetic level, since a screen of a transposon library for non-pigmented mutants once again mapped all insertions to the *cyl* locus (Spellerberg *et al*., 2000). Targeted mutagenesis of *cyl*E performed to knock out the βH/C phenotype also invariably knocked out expression of the orange pigment, and pigmentation returned when *cyl*E was used to complement the mutant *in trans* (Pritzlaff *et al*., 2001). Among clinical isolates, there is a direct correlation between the degree of pigmentation of the GBS colony and the strength of the surrounding zone of β-hemolysis (Merritt and Jacobs, 1978; Tapsall, 1986, 1987). And whether they were generated by chemical, transposon, or targeted mutagenesis, all NH GBS derivatives lack pigmentation and all HH derivatives display enhanced pigmentation (Nizet *et al*., 1996; Spellerberg *et al*., 1999; Spellerberg *et al*., 2000; Pritzlaff *et al*., 2001).

One should not conclude that one gene (*cyl*E) encodes both the βH/C and pigment, since all known carotenoid pigments are assembled in a series of enzymatic steps. Neither of the published complete GBS genome sequences (Glaser *et al*., 2002; Tettelin *et al*., 2002) contain homologues of the enzymes typical of bacterial carotenogenesis (e.g., phytoene synthases, phytoene or carotene dehydrogenases, or lycopene cyclases); thus, the GBS pigment would appear to be the product of a unique biosynthetic pathway. The GBS pigment was recently found to play a role in pathogenesis by virtue of antioxidant properties consistent with the known oxygen radical scavenging potential of carotenoids. The pigment conferred protection to GBS against the antimicrobial effects of hydrogen peroxide, singlet oxygen, superoxide, and hypochlorite, all of which play a role in the oxidative burst-killing mechanism of host phagocytic cells (Liu *et al*., 2004). As a consequence, wild-type (WT) GBS has enhanced intracellular survival compared to non-pigmented, NH Δ*cyl*E mutants in neutrophil and macrophage killing assays, a difference that persists even when DPPC is added to block βH/C activity (Liu *et al*., 2004).

# BIOLOGICAL EFFECTS

## Cytolytic injury to eukaryotic cells

GBS βh/c has been demonstrated to lyse several different cell types, including sheep erythrocytes, murine

fibroblasts (Tapsall and Phillips, 1991), and human lung epithelial cells (Nizet *et al*., 1996; Doran *et al*., 2002), lung endothelial cells (Gibson *et al*., 1999), brain endothelial cells (Nizet *et al*., 1997b), and macrophages (Ring *et al*., 2000; Liu *et al*., 2004). Electron microscopic examination of GBS βh/c-hemolysin-induced injury to lung epithelial cells is consistent with the action of a pore-forming toxin (Figure 43.2). Discrete membrane disruptions, cellular swelling, loss of intracytoplasmic density, changes in subcellular organelles and chromatin are seen that appear to reflect entry of water into the cell and hypo-osmotic damage (Nizet *et al*., 1996). The GBS βh/c contributes to a loss of integrity in the tight junctions of polar lung endothelial cell monolayers, allowing passive flux of macromolecules across tight junctions (Gibson *et al*., 1999). There exist no reports to date of any eukaryotic cell types resistant to the cytotoxic effects of the GBS βh/c.

## Triggering of cellular apopotosis

The clumping of nuclear chromatin observed in epithelial cells injured by GBS βh/c is reminiscent of the pattern observed with apoptosis or programmed cell death. Live GBS has been shown to induce apoptosis in macrophages (Fettucciari *et al*., 2000; Ulett *et al*., 2003; Liu *et al*., 2004), splenocytes (Liu *et al*., 2004), and hepatocytes (Ring *et al*., 2002a), and several investigators have explored a potential linkage of the βH/C to this form of induced cell death.

Fettucciari and co-workers described a hemolytic GBS strain that upon incubation with murine peritoneal macrophages, induced apoptosis of the host cells within 24 hr (Fettucciari *et al*., 2000). Membrane permeability changes were noted as early as 2 hr in 70% of cells by propidium iodide exclusion studies, and progression to apoptosis was demonstrated by electron microscopy, DNA fragmentation assay, and *in situ* TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick end labeling) staining. Under conditions that either inhibited βH/C production (high glucose concentrations) or inhibited βH/C activity (addition of DPPC), apoptosis was abrogated. These observations led the authors to suggest that the βH/C was responsible for macrophage apoptosis. Subsequently, Ulett *et al*. compared the ability of WT or Δ*cyl*E mutant GBS to induce apoptosis in J774 macrophages (Ulett *et al*., 2003). Under their experimental conditions, the viability of macrophages exposed to each strain was similar, but significantly less than that of macrophages exposed to GBS that had been grown in high-glucose media. The authors proposed that macrophage apoptosis induced by GBS was independent of βH/C, and instead reflected the action of a GBS factor coregulated along with βH/C by glucose.

Our recent study attempted to shed light on these discrepancies by assessing macrophage apoptosis induced by GBS strains expressing different levels of βH/C and their corresponding NH Δ*cyl*E allelic exchange mutants (Liu *et al*., 2004). Apoptosis of murine peritoneal macrophage apoptosis was noted only with a strong hemolytic WT strain, but not with a weak hemolytic strain or either of the Δ*cyl*E mutants. The level of apoptosis was more prominent at higher bacterial inocula and could be inhibited with DPPC. Incubation of macrophages with an increasing dose of crude βH/C extract led to a dose-dependent increase

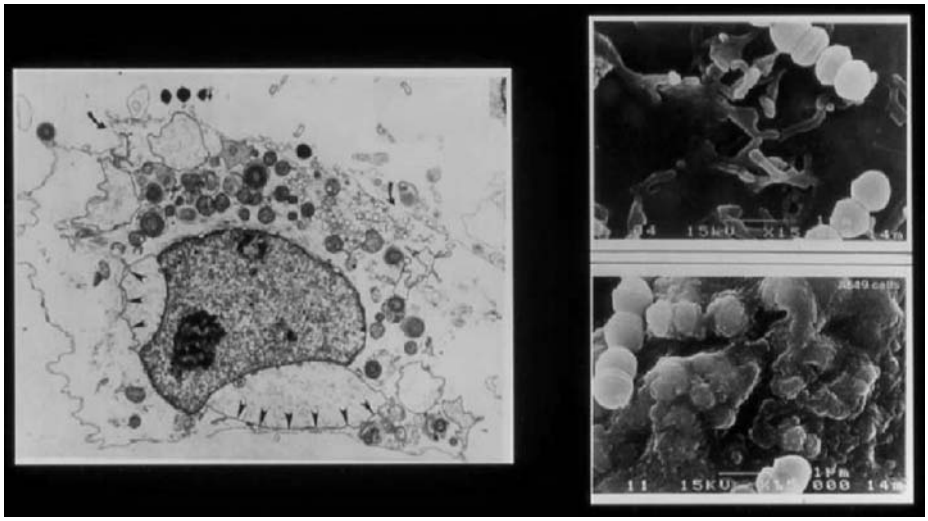


**FIGURE 43.2** GBS β-hemolysin/cytolysin mediated injury to cultured human lung epithelial cells. At left, transmission electron microscopy shows membrane pore formation, swelling of cell contents, and clumping of nuclear chromatin. At right, the normal villous architecture of the lung epithelial surface (top) seen with a weakly hemolytic GBS isolate is abolished to swelling and bleb formation upon exposure to a strongly hemolytic isolate (below) (Nizet *et al*., 1996).

in the number of TUNEL positive-staining macrophages. In mice challenged intravenously with GBS, apoptosis was abundantly evident among splenocytes in those animals infected with a strongly hemolytic WT GBS strain but not in mice challenged with its isogenic Δ*cyl*E mutant (Liu *et al*., 2004). In a study of septic liver injury in rabbits challenged intravenously with WT and βh/c mutant GBS, positive TUNEL staining and activation of caspase-3 in hepatocytes were also correlated to toxin production (Ring *et al*., 2002a). The balance of experimental data suggest that GBS induces apoptosis by βH/C-dependent and βH/C-independent mechanisms, with the toxin playing a greater role at higher bacterial concentrations and in the case of strongly hemolytic strains.

The mechanism of GBS βH/C-induced apoptosis may reflect subcytolytic perturbance of membrane integrity and influx of calcium ion, as the degree of apoptosis is predictably modulated through addition of excess cation to the media or chelation with EGTA (Fettucciari *et al*., 2000). More detailed analyses by Fettucciari and colleagues pointed to the involvement of c-Jun $NH_2$ terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), three members of mitogen-activated protein kinases (MAPKs) family, in GBS-induced macrophage apoptosis (Fettucciari *et al*., 2003). The authors demonstrated that during induction of apoptosis, WT hemolytic GBS stimulates a strong and persistent activation of JNK and p38 with concomitant inhibition of ERK. In contrast, use of a NH GBS strain induces only a transient activation of JNK, p38, and ERK MAPKs with no resultant apoptosis.

## Cell activation and proinflammatory signaling

Membrane pore formation and resulting ion fluxes likely result in activation of signaling cascades within host cells. Pore formation may also facilitate introduction of other effector molecules that directly activate certain host signaling pathways, as has been documented both in enterobactereciae (Cornelis and Van Gijsegem, 2000) and with streptolysin O of GAS (Madden *et al*., 2001). A pore-forming toxin of *Streptococcus pneumoniae*, pneumolysin, interacts with Toll-like receptor 4 (TLR-4) to stimulate innate inflammatory responses (Malley *et al*., 2003). Although it is unknown whether any or all of these potential mechanisms apply, the GBS βH/C has been shown to activate certain inflammatory response genes in target cells.

In human lung epithelial cells, the GBS βH/C stimulates gene transcription and release of interleukin-8 (IL-8), a potent neutrophil chemokine (Doran *et al*., 2002). The βH/C also promotes GBS intracellular invasion of lung epithelium, and both IL-8 release and invasion can be inhibited by the addition of DPPC (Doran *et al*., 2002). In human brain microvascular endothelial cells, oligonucleotide microarray analysis was employed to assess the transcriptional response of the blood-brain barrier to GBS and to specific GBS virulence factors (Doran *et al*., 2003). It was observed that GBS infection of the brain endothelium induced a highly specific and coordinate set of genes that act to orchestrate neutrophil recruitment and activation. These included the chemokines IL-8, Groα and Groβ, the neutrophil stimulating growth factor GM-CSF, the antiapoptotic factor Mcl-1, and the endothelial neutrophil receptor and activating molecular ICAM-1. Strikingly, infection of the endothelial cells with a Δβh/c mutant resulted in a marked reduction in expression of these genes involved in the neutrophil inflammatory response. Cell-free bacterial supernatants containing the βh/c toxin induced IL-8 release, identifying the toxin as the principal provocative factor for blood-brain barrier inflammatory activation. Migration of human neutrophils across polar brain endothelial cell monolayers was stimulated by the βh/c through a process involving IL-8 and ICAM-1 (Doran *et al*., 2003).

In murine macrophages, βH/C induces the expression of nitric oxide synthase (iNOS) and the release of NO, a potent vasodilator proximal mediator of the sepsis cascade (Ring *et al*., 2000). Stimulation of NO release by βH/C appears to depend on protein tyrosine kinases and NF-κB, suggesting the involvement of intracellular pathways similar to those that mediate lipopolysaccharide-induced iNOS activation (Ring *et al*., 2000). The induction of NO by GBS βH/C likely requires a coactivating factor be present on the cell surface, as a cell-free βH/C extract does not trigger NO production. In non-activated macrophages, synergistic activation by GBS cell wall components and βH/C is necessary to induce a NO response, which indicates that both factors cooperate to substitute for the priming signal typically provided by IFNγ (Ring *et al*., 2002b).

In summary, the GBS βH/C is a potent toxin with proven cytolytic activity against a wide variety of cell types encountered during the various steps of human disease pathogenesis. At subcytolytic doses, the GBS βH/C exerts a variety of proinvasive, proinflammatory, and proapoptotic effects on target cells. Therefore, it is logical to hypothesize that such effects would have deleterious consequences on the infected host. The contribution of the βH/C toxin to GBS pathogenesis has been investigated in several *in vivo* models of invasive disease and is reviewed in the next section.

## βH/C IN THE PATHOGENESIS OF GBS NEONATAL INFECTION

Pneumonia, sepsis, and meningitis are the three most common clinical manifestations of invasive GBS infection in human newborns. In the more frequent instance of early-onset infection, the infant directly aspirates an inoculum of GBS from contaminated amniotic or vaginal fluids *in utero* or during passage through the birth canal. These infants present with respiratory signs indicative of an initial pulmonary focus of infection, and the GBS quickly spreads to the bloodstream to produce acute septicemia. Late-onset GBS infections can develop in infants up to several months of age, and are suspected to reflect mucosal colonization with the bacterium and subsequent invasion into the bloodstream. These infections often have a subacute presentation with a high rate of dissemination to end organs, such as the central nervous system to produce meningitis, or to skeletal foci to produce osteomyelitis or septic arthritis (Nizet and Rubens, 2000; Doran and Nizet, 2004). Because clear βH/C activity is apparent in the vast majority of GBS isolated from clinical sources, several efforts have been directed at identifying the role of this toxin in disease pathogenesis. The availability of isogenic transposon or allelic exchange mutants with altered βH/C phenotype has allowed direct assessment of the toxin in several small animal models of GBS infection.

### Pneumonia

The first *in vivo* demonstration of a βH/C effect on lung pathology was accomplished using chemically derived NH and HH GBS mutants (Wennerstrom *et al*., 1985). These researchers used intranasal inoculation to challenge mice with GBS, and found that increased βH/C activity was associated with greater degrees of cellular injury, as measured by lactate dehydrogenase release into the bronchalveolar fluid, and a decreased $LD_{50}$ and earlier time to death for a given inoculum. Similar results were observed upon transthoracic challenge of neonatal rats with WT GBS and isogenic HH and NH transposon mutants. $LD_{50}$ at 48 hr for the NH mutant was 1,000-fold greater than $LD_{50}$ for either the WT strain or HH mutant, and the HH mutant lead to death of the infected animal more rapidly than the WT strain (Nizet *et al*., 1997a).

Most recently, pathophysiology associated with βH/C was investigated in a neonatal rabbit model of pneumonia induced by direct intracheal injection of GBS bacteria (Hensler *et al*., 2005). Animals infected with wild-type (WT) GBS developed focal pneumonia and by 18 h had 100-fold more bacteria in lung tissues than rabbits infected with the Δβh/c mutant. Mortality, development of bacteremia, and mean bacterial blood counts were all significantly higher in the animals challenged with WT GBS compared to the Δβh/c mutant. Lung compliance during mechanical ventilation was impaired upon infection with WT GBS but not with the Δβh/c strain. This work provided *in vivo* evidence for a critical role of the βh/c toxin in GBS neonatal pneumonia and the breakdown of the pulmonary barrier to systemic infection.

When a heavy inoculum of GBS is aspirated by the newborn, βH/C is likely to contribute to pneumonia by direct lysis of lung epithelial and pulmonary endothelial cells and perhaps alveolar macrophages, contributing to barrier breakdown and systemic dissemination (Nizet *et al*., 1996; Liu *et al*., 2004; Hensler *et al*., 2005). Supporting this hypothesis are the *in vitro* studies demonstrating a direct correlation of GBS βH/C activity to lung cellular injury as measured by LDH release, trypan blue nuclear staining, albumin flux, and ultrastructural examination (Nizet *et al*., 1996; Gibson *et al*., 1999). The stimulation of IL-8 release by the βh/c may contribute to neutrophilic inflammation seen in neonatal pneumonia, whereas the toxin's promotion of GBS cellular invasion may facilitate bloodstream entry (Doran *et al*., 2002). The very same phospholipid DPPC that inhibits βH/C-induced cytotoxicity (Nizet *et al*., 1996), apoptosis (Fettucciari *et al*., 2000; Liu *et al*., 2004), and proinflammatory effects (Doran *et al*., 2002) is also the major constituent of human pulmonary surfactant (70% by weight). Deficiency in pulmonary surfactant may explain the markedly increased risk of premature, low-birth weight infants who suffer severe GBS pneumonia and sepsis (Tapsall and Phillips, 1991; Doran and Nizet, 2004).

### Septicemia

Sepsis occurs when GBS successfully breach the primary barrier of the body to produce clinical symptoms of hemodynamic instability and organ dysfunction. The first evidence that the GBS βH/C could contribute to the pathogenesis of septicemia was provided by Griffiths and Rhee, who found that systemic administration of a GBS βH/C extract to rabbits produced a significant acute hypotensive reaction and a limited number of deaths due to shock (Griffiths and Rhee, 1992). In parallel experiments, injection of GAS streptolysin S extract had no such effect on hemodynamics. In comparison of WT GBS and βH/C mutants in

murine intravenous challenge models of septicemia, the toxin is found to contribute markedly to an overall mortality and rapidity of death (Puliti *et al*., 2000; Liu *et al*., 2004). These changes are likely to reflect a combination of the antiphagocytic and proinflammatory properties of the toxin. The ability of GAS βh/c to lyse host neutrophils and macrophages and/or trigger their apoptosis leads to enhanced survival of the organism on encounter with the phagocytic cells (Liu *et al*., 2004). Systemic release of the proinflammatory cytokine IL-6 is greatly increased in response to a hemolytic GBS strain compared to its isogenic NH mutant (Puliti *et al*., 2000).

The most detailed analysis of GBS βH/C in the pathogenesis of septicemia was accomplished using WT GBS and isogenic mutants in the adult rabbit model (Ring *et al*., 2002a). Mortality was highest in rabbits challenged with an HH mutant (67%), compared to animals infected with the weakly hemolytic parent strain (27%) or the NH mutant (0%). Corresponding changes in bacterial counts in the blood and mean arterial pressure confirmed the association of βH/C expression with resistance to phagocytic clearance and induction of sepsis symptomatology. Histopathologically, disseminated septic microabscesses surrounded by necrotic foci were found exclusively in the livers of animals infected with the HH mutant, and they also exhibited a 20-fold increase in serum transaminase levels and evidence of hepatocyte necrosis and apoptosis. Together, the work provided clear evidence that GBS βH/C plays a crucial role in the pathophysiology of GBS sepsis by inducing liver failure and high mortality (Ring *et al*., 2002a).

## Meningitis

A feared but not infrequent manifestation of invasive GBS disease is meningitis. Meningitis is a significant cause of mortality in GBS disease and 25–40% of infants may suffer permanent neurological sequelae, such as hearing loss, cognitive delay, seizures, or cerebral palsy. In animal models, the risk of meningitis can be correlated to the magnitude and duration of GBS bacteremia (Ferrieri *et al*., 1980), and the known effects of βH/C in blocking phagocytic clearance (Liu *et al*., 2004) would be predicted to increase the chance of meningitis on this account alone. GBS also have the ability to invade and transcytose human brain endothelial cells (Nizet *et al*., 1997b), the single-cell layer that comprises the majority of the blood-brain barrier. The ability of the βH/C to stimulate GBS intracellular invasion (Doran *et al*., 2002) could be seen as a second potential factor promoting the development of meningitis. Finally, GBS has been shown to activate inflammatory gene expression in human brain endothelial cells and to promote the consequent recruitment, activation, and transcytosis of neutrophils (Doran *et al*., 2003). Inflammatory damage to the blood-brain barrier induced by the βH/C therefore represents a third mechanism by which the toxin could promote the development of meningitis.

Consistent with the above mechanisms identified by *in vitro* experiments, mice challenged *in vivo* with an isogenic ΔβH/C mutant experience less blood-brain barrier penetration and suffer fewer cases of meningitis than mice challenged with the WT GBS parent strain (Doran *et al*., 2003). Bacterial counts in brain tissue were significantly higher in the animals challenged with WT GBS, and histopathologic studies confirmed meningeal thickening, thrombosis of meningeal vessels, leukocytic infiltration, and areas of bacterial microabscess formation in the brain parenchyma, changes generally absent in mice challenged with the ΔβH/C mutant. Additional studies performed by intrathecal challenge of rats with GBS WT and ΔβH/C mutant strains have shown a direct correlation of toxin expression to apoptosis of hippocampal neurons (J. Weber and V. Nizet, unpublished), a point of clinical concern given the limited plasticity of neuronal cells for regeneration.

## Potential therapeutics

Because the βH/C contributes directly to disease pathogenesis through direct cellular injury, resistance to immune clearance, and activation of local and systemic inflammatory responses, the toxin constitutes an attractive target for adjunctive therapy of infants with GBS infection. Neutralizing antibody to the βH/C does not arise in the course of natural infection (Todd, 1934), and no specific antisera were detected upon repeated injection of partially purified βH/C preparations in rabbits for three months (Dal and Monteil, 1983). Progress in development of antibodies may benefit from more detailed knowledge of protein structure, as a synthetic peptide approach has recently been successful in generating inhibitory antibodies against the naturally non-immunogenic SLS toxin of GAS (Carr *et al*., 2001; Dale *et al*., 2002).

An alternative therapeutic modality to GBS infection may involve the use of phospholipid inhibitors to inactivate the βH/C. DPPC has been shown in many *in vitro* studies to be highly effective in blocking lysis of erythrocytes, fibroblasts, epithelial cells, and macrophages (Tapsall and Phillips, 1991; Nizet *et al*., 1996; Liu and Nizet, 2004). Likewise, cytokine secretion, apoptosis, and cellular invasion promoted by the GBS βH/C can also be prevented using this inhibitor

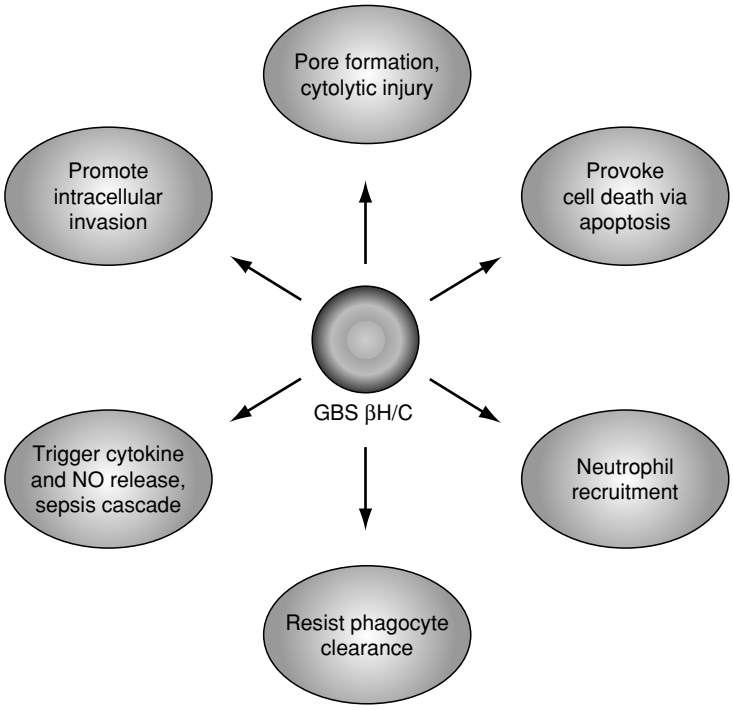



**FIGURE 43.3** Schematic representation of biological activities attributed to the GBS β-hemolysin/cytolysin, a multifunctional toxin and virulence factor in the pathogenesis of invasive infection.

(Fettucciari *et al*., 2000; Doran *et al*., 2002; Liu *et al*., 2004). DPPC is a major component of surfactant, which is used therapeutically to restore lung compliance in premature neonates. Indeed, experiments in a ventilated premature rabbit model of GBS pneumonia and retrospective analysis of clinical data from human neonates with early onset GBS suggest beneficial effects of surfactant therapy against GBS-induced lung injury (Herting *et al*., 1997; Herting *et al*., 2000). Recent data identifying a key role of the βH/C in newborn pneumonia and lung injury (Hensler *et al*., 2005) may provide a key molecular mechanism explaining the therapeutic efficacy of surfactant replacement in terms of DPPC inhibition of the toxin.

## CONCLUSION

Fueled by technological innovations in streptococcal genetics, the past decade has witnessed a resurgence of research into the biology and function of the GBS βH/C. The role of the βH/C as a virulence factor in disease pathogenesis has been confirmed in many animal models of GBS infection, and several unique properties of the toxin have been characterized. It is apparent that the βH/C is a multifunctional molecule, capable of exerting cytolytic, proapoptotic, proinvasive, proinflammatory, or antiphagocytic effects on a variety of target cells (Figure 43.3). At the biochemical level, select signaling pathways triggered by the βH/C are beginning to be unraveled. Yet the basic structural properties of the toxin remain poorly characterized because of difficulties presented in purification of this labile and apparently non-immunogenic toxin. Determination of the structure of the mature protein toxin represents the overriding challenge for the future of GBS βH/C research.

## ACKNOWLEDGMENTS

G.Y.L. is a Howard Hughes Medical Institute Postdoctoral Fellow and V.N. is an Edward Mallinckrodt, Jr. Foundation Scholar.

## REFERENCES

Atwood, K.C. and Norman, A. (1949). On the interpretation of multihit survival curves. *Proc. Natl. Acad. Sci. USA* **35,** 696–702.

Berner, R. (2004). Significance, management, and prevention of *Streptococcus agalactiae* infection during the perinatal period. *Expert. Rev. Anti. Infect. Ther*. **2,** 427–437.

Bernheimer, A.W. (1947). Comparative kinetics of hemolysis induced by bacterial and other hemoysins. *J. Gen. Physiolol*. **30,** 337–353.

Blancas, D., Santin, M., Olmo, M., Alcaide, F., Carratala, J. and Gudiol, F. (2004). Group B streptococcal disease in non-pregnant adults: incidence, clinical characteristics, and outcome. *Eur. J. Clin. Microbiol. Infect. Dis*. **23,** 168–173.

Carr, A., Sledjeski, D.D., Podbielski, A., Boyle, M.D. and Kreikemeyer, B. (2001). Similarities between complement-mediated and streptolysin S-mediated hemolysis. *J. Biol. Chem*. **276,** 41790–41796.

Cornelis, G.R. and Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annu. Rev. Microbiol*. **54,** 735–774.

Dal, M.C. and Monteil, H. (1983). Hemolysin produced by group B *Streptococcus agalactiae. FEMS Microbiol. Lett*. **16,** 89–94.

Dale, J.B., Chiang, E.Y., Hasty, D.L. and Courtney, H.S. (2002). Antibodies against a synthetic peptide of SagA neutralize the cytolytic activity of streptolysin S from group A streptococci. *Infect. Immun*. **70,** 2166–2170.

Doran, K.S., Chang, J.C., Benoit, V.M., Eckmann, L. and Nizet, V. (2002). Group B streptococcal beta-hemolysin/cytolysin promotes invasion of human lung epithelial cells and the release of interleukin-8. *J. Infect. Dis*. **185,** 196–203.

Doran, K.S., Liu, G.Y. and Nizet, V. (2003). Group B streptococcal β-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest*. **112,** 736–744.

Doran, K.S. and Nizet, V. (2004). Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. *Mol. Microbiol*. **54,** 23–31.

Duncan, J.L. and Mason, L. (1976). Characteristics of streptolysin S hemolysis. *Infect. Immun*. **14,** 77–82.

Felmlee, T., Pellett, S. and Welch, R.A. (1985). Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. *J. Bacteriol*. **163,** 94–105.

Ferrieri, P. (1982). *Basic Concepts of Streptococci and Streptococcal Diseases*. Reedbooks: Surrey.

Ferrieri, P., Burke, B. and Nelson, J. (1980). Production of bacteremia and meningitis in infant rats with group B streptococcal serotypes. *Infect. Immun*. **27,** 1023–1032.

Fettucciari, K., Fetriconi, I., Bartoli, A., Rossi, R. and Marconi, P. (2003). Involvement of mitogen-activated protein kinases in group B *Streptococcus*-induced macrophage apoptosis. *Pharmacol. Res*. **47,** 355–362.

Fettucciari, K., Rosati, E., Scaringi, L., Cornacchione, P., Migliorati, G., Sabatini, R., Fetriconi, I., Rossi, R. and Marconi, P. (2000). Group B *Streptococcus* induces apoptosis in macrophages. *J. Immunol*. **165,** 3923–3933.

Gibson, R.L., Nizet, V. and Rubens, C.E. (1999). Group B streptococcal beta-hemolysin promotes injury of lung microvascular endothelial cells. *Pediatr. Res*. **45,** 626–634.

Gilmore, M.S., Segarra, R.A. and Booth, M.C. (1990). An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. *Infect. Immun*. **58,** 3914–3923.

Ginsburg, I. (1970). Streptolysin S. In: *Microbial Toxins, Vol. 3. Bacterial Protein Toxins* (eds. T.C. Montiel, S. Kadis, and S.J. Ajl), pp. 97–176. Academic Press, New York.

Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T., Zouine, M., Couve, E., Lalioui, L., Poyart, C., Trieu-Cuot, P. and Kunst, F. (2002). Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol*. **45,** 1499–1513.

Glaser, P., Sakamoto, H., Bellalou, J., Ullmann, A. and Danchin, A. (1988). Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-hemolysin bifunctional protein of Bordetella pertussis. *Embo J*. **7,** 3997–4004.

Griffiths, B.B. and Rhee, H. (1992). Effects of hemolysins of groups A and B streptococci on cardiovascular system. *Microbios* **69,** 17–27.

Hensler, M.E., Liu, G.Y., Sobczak, S., Bernirshcke, K., Nizet, V. and Heldt, G.P. (2005). Virulence role of the group B streptococcal β-hemolysin/cytolysin in a neonatal rabbit model of early onset pulmonary infection. *J. Infect. Dis*. **191,** 1287–1291.

Herting, E., Gefeller, O., Land, M., van Sonderen, L., Harms, K. and Robertson, B. (2000). Surfactant treatment of neonates with respiratory failure and group B streptococcal infection. Members of the Collaborative European Multicenter Study Group. *Pediatrics* **106,** 957–964; discussion 1135.

Herting, E., Sun, B., Jarstrand, C., Curstedt, T. and Robertson, B. (1997). Surfactant improves lung function and mitigates bacterial growth in immature, ventilated rabbits with experimentally-induced neonatal group B streptococcal pneumonia. *Arch. Dis. Child Fetal Neonatal. Ed*. **76,** F3–8.

Inoue, K., Akiyama, Y., Kinoshita, T., Higashi, Y. and Amano, T. (1976). Evidence for a one-hit theory in the immune bactericidal reaction and demonstration of a multihit response for hemolysis by streptolysin O and *Clostridium perfringens* theta-toxin. *Infect. Immun*. **13,** 337–344.

Lamy, M.-C., Zouine, M., Fert, J., Vergassola, M., Couve, E., Pellegrini, E., Glaser, P., Kunst, F., Msadek, T., Trieu-Cuot, P. and Poyart, C. (2004). CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. *Mol Microbiol*. **54,** 1250–1268.

Liu, G.Y., Doran, K.S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L. and Nizet, V. (2004). Sword and shield: Linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proc. Natl. Acad. Sci. USA* **101,** 14491–14496.

Liu, G.Y. and Nizet, V. (2004). Extracellular virulence factors of group B streptococci. *Front Biosci*. **9,** 1794–1802.

Madden, J.C., Ruiz, N. and Caparon, M. (2001). Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152.

Malley, R., Henneke, P., Morse, S.C., Cieslewicz, M.J., Lipsitch, M., Thompson, C.M., Kurt-Jones, E., Paton, J.C., Wessels, M.R. and Golenbock, D.T. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc. Natl. Acad. Sci. USA* **100,** 1966–1971.

Marchlewicz, B.A. and Duncan, J.L. (1980). Properties of a hemolysin produced by group B streptococci. *Infect. Immun*. **30,** 805–813.

Marchlewicz, B.A. and Duncan, J.L. (1981). Lysis of erythrocytes by a hemolysin produced by a group B *Streptococcus* sp. *Infect. Immun*. **34,** 787–794.

Merritt, K. and Jacobs, N.J. (1978). Characterization and incidence of pigment production by human clinical group B streptococci. *J. Clin. Microbiol*. **8,** 105–107.

Morbidoni, H.R., de Mendoza, D. and Cronan, J.E., Jr. (1996). *Bacillus subtilis* acyl carrier protein is encoded in a cluster of lipid biosynthesis genes. *J. Bacteriol*. **178,** 4794–4800.

Nizet, V. (2002). Streptococcal β-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol*. **10,** 575–580.

Nizet, V., Gibson, R.L., Chi, E.Y., Framson, P.E., Hulse, M. and Rubens, C.E. (1996). Group B streptococcal beta-hemolysin expression is associated with injury of lung epithelial cells. *Infect. Immun*. **64,** 3818–3826.

Nizet, V., Gibson, R.L. and Rubens, C.E. (1997a). The role of group B streptococci β-hemolysin expression in newborn lung injury. *Adv. Exp. Med. Biol*. **418,** 627–630.

Nizet, V., Kim, K.S., Stins, M., Jonas, M., Chi, E.Y., Nguyen, D. and Rubens, C.E. (1997b). Invasion of brain microvascular endothelial cells by group B streptococci. *Infect. Immun*. **65,** 5074–5081.

Nizet, V. and Rubens, C.E. (2000). Pathogenic mechanisms and virulence factors of group B streptococci. In: *The Gram-Positive Pathogens* (ed. R.N. VA Fischetti, JJ Ferretti, DA Portnoy, JI Rood), pp. 125–136. ASM Press, Washington, D.C.

Platt, M.W. (1995). *In vivo* hemolytic activity of group B *Streptococcus* is dependent on erythrocyte-bacteria contact and independent of a carrier molecule. *Curr. Microbiol*. **31,** 5–9.

Pritzlaff, C.A., Chang, J.C., Kuo, S.P., Tamura, G.S., Rubens, C.E. and Nizet, V. (2001). Genetic basis for the β-hemolytic/cytolytic activity of group B *Streptococcus*. *Mol. Microbiol*. **39,** 236–247.

Puliti, M., Nizet, V., von Hunolstein, C., Bistoni, F., Mosci, P., Orefici, G. and Tissi, L. (2000). Severity of group B streptococcal arthritis is correlated with beta-hemolysin expression. *J. Infect. Dis*. **182,** 824–832.

Rawlings, M. and Cronan, J.E., Jr. (1992). The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. *J. Biol. Chem*. **267,** 5751–5754.

Ring, A., Braun, J.S., Nizet, V., Stremmel, W. and Shenep, J.L. (2000). Group B streptococcal beta-hemolysin induces nitric oxide production in murine macrophages. *J. Infect. Dis*. **182,** 150–157.

Ring, A., Braun, J.S., Pohl, J., Nizet, V., Stremmel, W. and Shenep, J.L. (2002a). Group B streptococcal beta-hemolysin induces mortality and liver injury in experimental sepsis. *J. Infect. Dis*. **185,** 1745–1753.

Ring, A., Depnering, C., Pohl, J., Nizet, V., Shenep, J.L. and Stremmel, W. (2002b). Synergistic action of nitric oxide release from murine macrophages caused by group B streptococcal cell wall and beta-hemolysin/cytolysin. *J. Infect. Dis*. **186,** 1518–1521.

Ross, R.A., Madoff, L.C. and Paoletti, L.C. (1999). Regulation of cell component production by growth rate in the group B *Streptococcus*. *J. Bacteriol*. **181,** 5389–5394.

Shen, Z. and Byers, D.M. (1996). Isolation of *Vibrio harveyi* acyl carrier protein and the *fab*G, *acp*P, and *fab*F genes involved in fatty acid biosynthesis. *J. Bacteriol*. **178,** 571–573.

Spellerberg, B., Martin, S., Brandt, C. and Lutticken, R. (2000). The *cyl* genes of *Streptococcus agalactiae* are involved in the production of pigment. *FEMS Microbiol. Lett*. **188,** 125–128.

Spellerberg, B., Pohl, B., Haase, G., Martin, S., Weber-Heynemann, J. and Lutticken, R. (1999). Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. *J. Bacteriol*. **181,** 3212–3219.

Tapsall, J.W. (1986). Pigment production by Lancefield group B streptococci (*Streptococcus agalactiae*). *J. Med. Microbiol*. **21,** 75–81.

Tapsall, J.W. (1987). Relationship between pigment production and hemolysin formation by Lancefield group B streptococci. *J. Med. Microbiol*. **24,** 83–87.

Tapsall, J.W. and Phillips, E.A. (1991). The hemolytic and cytolytic activity of group B streptococcal hemolysin and its possible role in early onset group B streptococcal disease. *Pathology* **23,** 139–144.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Eisen, J.A., Peterson, S., Wessels, M.R., Paulsen, I.T., Nelson, K.E., Margarit, I., Read, T.D., Madoff, L.C., Wolf, A.M., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., DeBoy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Lewis, M.R., Radune, D., Fedorova, N.B., Scanlan, D., Khouri, H., Mulligan, S., Carty, H.A., Cline, R.T., Van Aken, S.E., Gill, J., Scarselli, M., Mora, M., Iacobini, E.T., Brettoni, C., Galli, G., Mariani, M., Vegni, F., Maione, D., Rinaudo, D., Rappuoli, R., Telford, J.L., Kasper, D.L., Grandi, G. and Fraser, C.M. (2002). Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. *Proc. Natl. Acad. Sci. USA* **99,** 12391–12396.

Todd, E.W. (1934). A comparative serological study of streptolysins derived from human and animal infections with note on pneumococcal hemolysin, tetanolysin, and streptococcal toxin. *J. Pathol. Bacteriol*. **39,** 299–321.

Tsaihong, J. and Wennerstrom, D. (1983). Effect of carrier molecules on production and properties of extracellular hemolysin produced by *Streptococcus agalactiae*. *Curr. Microbiol*. **9,** 333–338.

Ulett, G.C., Bohnsack, J.F., Armstrong, J. and Adderson, E.E. (2003). Beta-hemolysin-independent induction of apoptosis of macrophages infected with serotype III group B *Streptococcus*. *J. Infect. Dis*. **188,** 1049–1053.

Weiser, J.N. and Rubens, C.E. (1987). Transposon mutagenesis of group B streptococcus beta-hemolysin biosynthesis. *Infect. Immun*. **55,** 2314–2316.

Wennerstrom, D.E., Tsaihong, J.C. and Crawford, J.T. (1985). Evaluation of the role of hemolysin and pigment in the pathogenesis of early onset group B streptococcal infection. In: *Recent Advances in Streptococci and Streptococcal Diseases* (eds. Y. Kimura, S. Kotami, and Y. Shiokowa, Bracknell), pp. 155–156. Reedbooks, U.K.

CHAPTER

# 44

# Hemolysins of *vibrio cholerae* and other *vibrio* species

*Sumio Shinoda and Shin-ichi Miyoshi*

## INTRODUCTION

Bacteria of the genus *Vibrio* are normal habitants of the aquatic environment, but the 11 species listed in Table 44.1 are believed to be human pathogens (Janda, 1988; Chakraborty, 1977). These species can be classified into two groups according to the types of diseases they cause: One group causes gastrointestinal infections and the other extraintestinal infections (Table 44.1). The pathogenic species produce various pathogenic factors including enterotoxin, hemolysin, cytotoxin, protease, siderophore, adhesive factor, and hemagglutinin, with hemolysin being the one most widely distributed in the pathogenic vibrios (Table 44.2). Although the most important pathogenic factor of *Vibrio cholerae* O1/O139 is cholera enterotoxin (CT), a hemolysin (El Tor hemolysin) produced by biovar *eltor* is also reported to cause diarrhea. A thermostable direct hemolysin (TDH) produced by *Vibrio parahaemolyticus* is also believed to be a major pathogenic component of the species. This article offers a review of recent information on hemolysins of pathogenic vibrios with special reference to the above two representatives.

## *VIBRO CHOLERAE* HEMOLYSINS

Of the currently recognized pathogenic vibrios, the species *V. cholerae* is the most extensively studied because it is the causative agent of the often lethal disease cholera. The total genome sequence of 1,072,314 bp of *V. cholerae* strain N16961 was published by Heidelberg *et al*. (2000). Cholera is characterized by copious uncontrolled purging of rice water stools leading to severe electrolyte depletion, dehydration, acidosis, shock, and, if left untreated, death. Characteristic signs and symptoms of severely dehydrated patients include an increase in pulse rate and a decrease in pulse volume, hypotension, an increase in respiratory rate accompanied by deep respiration, sunken eyes and cheeks, dry mucous membrane, decreased skin turgor, abdominal pain, a decrease in urine output, and thirst (Harvey *et al*., 1966).

*V. cholerae* is serologically classified by the major surface O antigen. Until the emergence of the O139 serogroup, only the O1 serogroup had been thought to be the etiologic agent of cholera. All strains that were identified as *V. cholerae* on the basis of biochemical tests but were negative for agglutination with O1 antiserum were referred to as non-O1 *V. cholerae*. The emergence of *V. cholerae* O139 Bengal as the second etiologic agent of cholera in October 1992 in the southern Indian coastal city of Madras (Ramamurthy *et al*., 1993; Albert *et al*., 1993) necessitated the redefinition of non-O1 *V. cholerae* to non-O1/non-O139 *V. cholerae*. The majority of non-O1/non-O139 strains do not produce cholera enterotoxin (CT) and are not associated with epidemic diarrhea, although non-O1/non-O139 strains are occasionally isolated from cases of diarrhea and a variety of extraintestinal infections, including wounds, ear, sputum, urine, and cerebrospinal fluid (Morris, 1990).

CT is the major toxin causing the severe watery diarrhea produced by *V. cholerae* O1 and O139 strains. Zonula occludens toxin (Zot) (Fasano, 1991) and accessory cholera toxin (Ace) (Trucksis *et al*., 1993) have also

**TABLE 44.1** Pathogenic species of the genus *Vibrio*

| Gastrointestinal diseases | Extraintestinal disease |
|---|---|
| *V. cholerae* O1 | *V. vulnificus* |
| *V. cholerae* O139 | *V. damsela* |
| *V. cholerae* non-O1/non-O139 | *V. alginolyticus* |
| *V. mimicus* | *V. cincinnatiensis* |
| *V. parahaemolyticus* | *V. metschnikovii* |
| *V. fluvialis* | |
| *V. furnissii* | |
| *V. hollisae* | |
| *V. metschnikovii* | |

been demonstrated to be enterotoxic factors of these two serogroups. *V. cholerae* non-O1/non-O139 strains have been reported to produce a 17 amino acid, heat-stable enterotoxin (NAG-ST) (Takeda *et al*., 1991), and the production of hemolysins has also been reported.

El Tor strains of *V. cholerae* O1 produce a hemolysin (El Tor hemolysin) that has a lytic effect on Vero and other mammalian cells in culture (Honda and Finkelstein, 1979). Some strains of *V. cholerae* non-O1/non-O139 produce a hemolysin (NAG hemolysin) that is indistinguishable biologically, physicochemically, and antigenically from El Tor hemolysin (Yamamoto *et al*., 1986). Hemolysins were purified from culture filtrates of the vibrios and their properties were investigated. Both hemolysins were indistinguishable in their molecular sizes (about 65 kDa), amino acid compositions, and immunological characteristics. As El Tor hemolysin and NAG hemolysin are thought to be the same hemolysin, they are abbreviated to VCC (*V. cholerae* cytolysin) in this chapter. VCC can cause bloody fluid accumulation in ligated rabbit ileal loops, in contrast to the watery fluid produced by CT (Ichinose *et al*., 1987); thus, VCC may play a role in the pathogenesis of gastroenteritis caused by *V. cholerae* strains.

**TABLE 44.2** Hemolysins produced by vibrios

| | Name or abbreviation | Remarks |
|---|---|---|
| 1. Hemolysins related to El Tor hemolysin | | |
| *V. cholerae* O1 biotype eltor | El Tor hemolysin (VCC) | 65kDa (64,864Da)[a], Two-step processing for mature protein, Colloid osmotic hemolysis, RIL[b] positive |
| *V. cholerae* non-O1/non-O139 | NAG hemolysin | Indistinguishable from El Tor hemolysin (biologically, physicochemically, immunologically) |
| *V. mimicus* | VMH | 63kDa (65,972Da), Two-step processing for mature protein, Colloid osmotic hemolysis, RIL positive, 76% homology with El Tor hemolysin gene |
| *V. fluvialis* | | 63kDa, Related to VCC immunologically and genetically |
| *V. furnissii* | | Indistinguishable from *V. fluvialis* hemolysin |
| *V. anguillarum* | | 84,260Da (Calculated from amino acid sequence deduced from open reading frame including signal peptide), 57% homology with El Tor hemolysin gene |
| 2. Hemolysin related to Vp-TDH | | |
| *V. parahaemolyticus* | | |
| KP positive strains | Vp-TDH | Dimer of 21kDa (18,496Da) subunit, Thermostable (heating at 100°C), Colloid osmotic hemolysis, RIL positive, Cardiotoxicity |
| KP negative but pathogenic strains | Vp-TRH | Closely related to Vp-TDH but thermolabile, Virulence factor in diarrheal cases due to KP-strains |
| *V. hollisae* | Vh-rTDH | Thermolabile |
| *V. cholerae* non-O1/non-O139 | NAG-rTDH | Thermostable |
| *V. mimicus* | Vm-rTDH | Thermostable |
| 3. Other hemolysins | | |
| *V. vulnificus* | VVH (vulnificolysin) | 50kDa (50,851Da), Colloid osmotic cell lysis, Inducing apoptosis, Temperature-independent binding to cholesterol (suspected binding site) |
| *V. damsela* | Damselysin | 69kDa, Phospholipase D |
| *V. metschnikovii* | | 50kDa, Colloid osmotic cell lysis |
| *V. parahaemolyticus* | LDH (TLH) | 43kDa (41,453Da) and 45kDa (42,794Da), Lecithin-dependent indirect hemolysis (Phospholipase A2/lisophospholipase) |
| | δ-VPH | Produced by transformed *E. coli* cell, but not found in *V. parahaemolyticus* culture |

[a] Molecular weight deduced from SDS PAGE and amino acid sequence in parenthesis
[b] Rabbit ileal loop test (a test for enterotoxic activity)

Cell vacuolation by VCC is thought to contribute to virulence of *V. cholerae*. Coelho *et al*. (2000) showed correlation between vacuolation of Vero cells and VCC production in *V. cholerae* O1 Amazonia variant, and Mitra *et al*. (2000) showed that vacuolation of HeLa cells caused by various serogroups of *V. cholerae* was the action of VCC. Figueroa-Arredondo *et al*. (2001) demonstrated that one-third of diarrhea-associated non-epidemic *V. cholerae* strains from Mexico caused vacuolation of cultured Vero cells. They cloned the gene for vacuolating activity and found it to correspond to *hlyA* encoding VCC. The effect on local intestinal inflammatory or other defense responses by perturbing intracellular trafficking or ion exchange is suggested.

Yamamoto *et al*. (1990a) cloned and sequenced the genes of VCC composed of *hlyA* and *hlyB*, in which *hlyA* is a structural gene of the hemolysin molecule (Figure 44.1) and *hlyB* is thought to play a role of chaperone. They demonstrated that the VCC is synthesized as an 82-kDa precursor form (prepro-HlyA) consisting of a signal peptide (25 amino acid residues) at the N-terminus, a pro-region (132 residues), and a mature region (584 residues) at the C-terminus. The mature form of VCC is produced by a two-step processing; during passage through the inner membrane, the signal peptide is cleaved off, followed immediately by extracellular secretion in which the pro-region is lost. This two-step processing is necessary to activate VCC (Yamamoto *et al*., 1990a; Hall and Drasar, 1990; Nagamune *et al*., 1996). Nagamune *et al*. (1997) suggested that the pro-region of the hemolysin functions as an intramolecular chaperone, which plays a role in

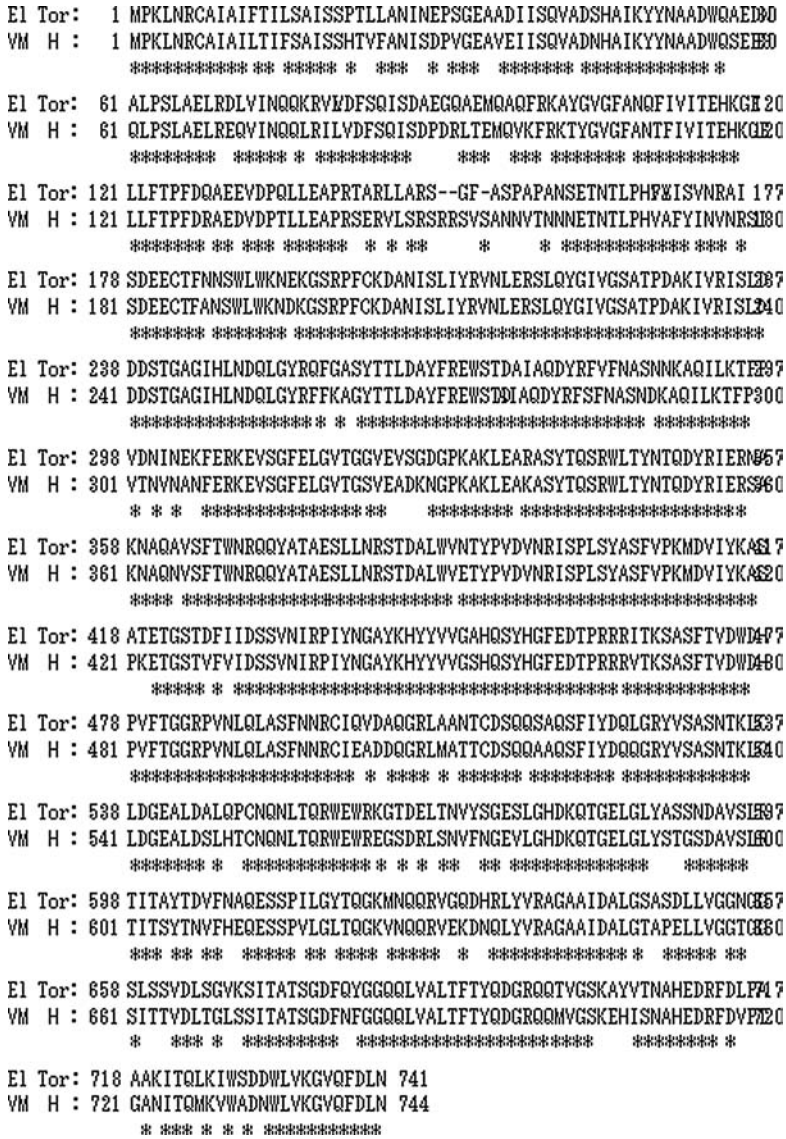



**FIGURE 44.1** DNA sequences of *hlyA*, gene encoding El Tor hemolysin (Yamamoto *et al*., 1990a) and VMH (Kim *et al*., 1997), whose GenBank Accession numbers are CAA36035 and AAB58399, respectively.

the correct folding of mature VCC (65 kDa). Valve *et al*. (2004) showed that 79 kDa pro-VCC also had binding ability to eukaryotic cells and was activated to mature form by the cellular metalloproteinase. A 50 kDa VCC is also found in culture filtrate of *V. cholerae*. Ikigai *et al*. (1999) showed that the 50 kDa VCC is in the C-terminus region of 65 kDa VCC and retains the essential part for oligomerization.

VCC is thought to act as a pore-forming toxin through its oligomer formation (Figure 44.2). The estimated size of the pore is 1.2 – 1.6 nm (Ikigai *et al*., 1996), and it is composed of pentameric oligomers (Zitzet *et al*., 1999). Cholesterol in the target cell membrane was reported to play an important role in the assembly of the toxin oligomers needed for pore formation by triggering oligomerization (Ikigai *et al*., 1996). Formation of channels in planar lipid phosphatidylcholine-cholesterol-bilayer membranes was also demonstrated (Ikigai *et al*., 1997). Saha and Banerjee (1997) showed carbohydrate-mediated regulation of interaction of VCC with erythrocyte and phospholipid vesicles. Menzl *et al*. (1997) showed some preferential movement of anions over cations through the pores at neutral pH by measuring zero-current membrane potentials, and has suggested that the VCC channel is moderately anion-selective. Although VCC is thought to be a unique pore-forming toxin differing from a paradigm such as staphylococcal α-hemolysin, amino acid sequence analysis and 3D structure modeling indicated similarity of the core sequence of VCC and α-hemolysin (Olson and Gouaux, 2003). Zhang *et al*. (1999) suggested that glycophorin, a snaloglycoprotein of erythrocyte membrane, is a receptor for VCC using a monoclonal antibody, which not only blocks the binding of VCC to human erythrocytes but also inhibits the VCC action.

The *tdh* gene encoding TDH of *V. cholerae* non-O1 (NAG-TDH) has 98.6% homology with *tdh*, which is thought to be the actual gene producing TDH of *Vibrio parahemolyticus* (Vp-TDH), so the property of NAG-TDH is quite similar to Vp-TDH (Baba *et al*., 1991).

A hemolysin having phospholipase C activity was purified from *V. cholerae* O139 strain and characterized (Pal *et al*., 1997). Fallarino *et al*. (2002) showed the existence of a new thermostable hemolysin of *V. cholerae*, Vc-δTH, which resembles the δ-VPH of *V. parahemolyticus* reported by Taniguchi *et al*. (1990).

## *VIBRIO PARAHEMOLYTICUS* HEMOLYSINS

*V. parahemolyticus* inhabits coastal and estuary waters and is recognized to cause gastroenteritis following consumption of seafood. This vibrio was discovered by Fujino *et al*. (1953) when an outbreak of food poisoning occurred in the southern suburbs of Osaka, Japan in October 1950.

The outstanding features of *V. parahemolyticus* infection are severe abdominal pain, diarrhea, nausea, vomiting, and mild fever, with headache, diarrhea (frequently bloody stools), and abdominal pain being the main symptoms (Miwatani and Takeda, 1976). The mean incubation period is 6 to 12 hours and diarrhea or soft stools persist for 4 to 7 days. The mortality rate is very low, although the first outbreak reported by Fujino *et al*. (1953) resulted in high mortality (20 victims out of 272 patients).

Although *V. parahemolyticus* is a natural inhabitant of seawater as shown above, only some of the isolates are pathogenic to humans. Miyamoto *et al*. (1969) reported that the hemolytic characteristic of this vibrio on a special agar medium (Wagatsuma's medium) correlated well with human pathogenicity. The hemolytic property on this medium was referred to as the Kanagawa phenomenon (KP) because its discoverers belonged to the Kanagawa Prefectural Public Health Laboratory. Sakazaki *et al*. (1968) examined the KP of the isolates

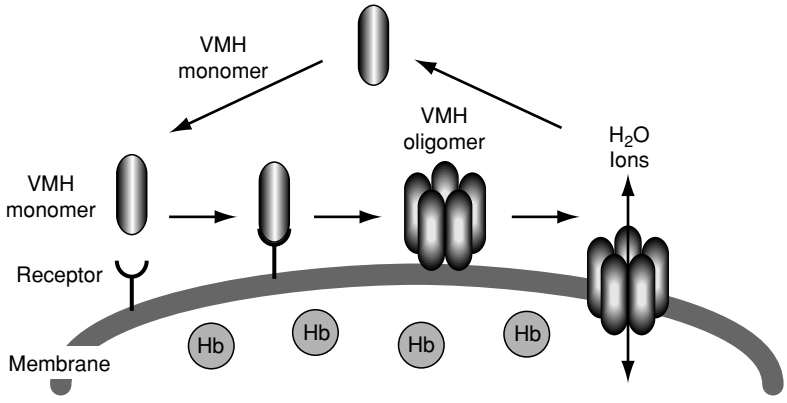



**FIGURE 44.2** Scheme for hemolysis induced by VMH (*Vibrio mimicus* hemolysin)

from human patients and from ocean fish and seawater and found a good correlation of KP and pathogenicity.

*V. parahemolyticus* is the major causative agent of food poisoning in Japan, and it is thought that the Japanese custom of eating raw fish, such as "sashimi" or "sushi," is the main reason for this high frequency. However, fish that are going to be eaten are handled very carefully in Japan, and are well-cleaned and kept at a low temperature. Therefore, it is suspected that a secondary source of contamination might be the utensils used to prepare the seafood.

The hemolysin causing KP is designated as TDH (thermostable direct hemolysin) because of its stability at 100°C. As similar hemolysins are found in vibrios other than *V. parahemolyticus*, the hemolysin of this species is often designated as Vp-TDH. Vp-TDH is a protein toxin having an approximate molecular size of 42 kDa composed of two identical 21 kDa subunits. The subunit consists of 165 amino acid residues with one disulfide bond near the carboxyl terminus (Tsunasawa *et al*., 1987). This result is in good agreement with that obtained from the nucleotide sequence of the gene encoding TDH (*tdh*).

Vp-TDH lyses red blood cells from various animal sources, one exception being the horse (Honda and Iida, 1993). The toxin is also cytotoxic to various cultured cells, such as HeLa cells, FL cells, and fetal mouse heart cells (Honda and Iida, 1993). In a rabbit ileal loop test, challenge of purified Vp-TDH at 100–250 μg/loop induced fluid accumulation (Honda and Iida, 1993; Miyamoto *et al*., 1980), which suggests that Vp-TDH is enterotoxic. The amount inducing fluid accumulation is higher than that of enterotoxins from other diarrheagenic bacteria; for example, 0.2 μg of CT is enough to induce this fluid accumulation. The contribution of Vp-TDH as the diarrheagenic factor has therefore been doubted. Other enterotoxic factors of *V. parahemolyticus*, such as one causing morphological change of Chinese hamster ovary (CHO) cells or cholera enterotoxin-like toxin immunologically cross-reactive in GM1-ganglioside ELISA, have also been suggested, but concrete evidence of the contribution of these factors has not been demonstrated. Extensive study of the rabbit ileal loop test with either Vp-TDH-producing or non-producing *V. parahemolyticus* strains suggested that only the former can induce fluid accumulation. To obtain evidence for the role of Vp-TDH in the enteropathogenicity of *V. parahemolyticus*, Nishibuchi *et al*. (1992) compared a KP-positive strain and its isogenic TDH-negative mutant in which both the *tdh1* and *tdh2* genes were specifically inactivated by successive allelic exchange procedures. Whole culture preparations of the parent strain gave positive fluid accumulation results in the rabbit ileal loop test, while the TDH-negative mutant induced no fluid accumulation in the model. Additional evidence for the enterotoxic activity of Vp-TDH was obtained with rabbit ileal tissue mounted in Ussing chambers, a sensitive technique for studying intestinal ion transport. Culture filtrates of the TDH-positive strain induced an increase in the short circuit current, whereas no such increase was observed with the isogenic TDH-negative strain (Nishibuchi *et al*. 1992). Returning the cloned *tdh* gene to the isogenic mutant complemented the mutation and restored the ability to increase the short circuit current. Thus, Vp-TDH is believed to be an important virulence factor in *V. parahemolyticus* gastroenteritis. Park *et al*. (2004), however, suggested the possibility of the existence of another factor for enterotoxicity. They constructed mutants of a pandemic strain of the vibrio lacking *tdhA* and/or *tdhS*, synonyms *tdh2* and *tdh1*, respectively, and showed that the mutant lacking both genes still showed partial enterotoxicity. They speculated that the reason for the contradiction with the results of Nishibuchi *et al*. (1992) might be the difference in strains. For example, a prepandemic strain was used in the former test, but a pandemic strain in the latter. The recent pandemic strain may have acquired a novel enterotoxic factor(s) in addition to TDH and TRH.

The mode of action of Vp-TDH has not yet been completely elucidated, though the hemolysis is thought to proceed in a colloid osmotic manner, which is shown in the section on VVH. Vp-TDH is believed to damage the erythrocyte membrane by acting as a pore-forming toxin (Honda *et al*., 1992). The functional pore size made by the hemolysin has been estimated to be 2 nm. One of the essential mechanisms in the damage that Vp-TDH inflicts during the hemolytic process is apparently the phosphorylation of the 25-kDa protein on the erythrocyte membrane. Yoh *et al*. (1996) found that TDH induced the phosphorylation of two proteins, 22.5 and 25 kDa, on membranes of human erythrocytes that are sensitive to TDH, but only the 22.5 kDa protein was phosphorylated on the membranes of horse erythrocytes that are insensitive. Furthermore, a mutant hemolysin, which retained binding ability but had lost hemolytic activity, also phosphorylated only the 22.5 kDa protein on human erythrocyte membranes. Zhang and Honda (1999) reported the disappearance of glyceraldehyde 3-phosphate dehydrogenase from the erythrocyte membrane by treatment with Vp-TDH and suggested a role of the enzyme in the hemolytic action.

Vp-TDH seems to cause hemolysis and to lyse cultured cells in a calcium-independent manner (Tang *et al*., 1994; Tang *et al*., 1995). A calcium-dependent chloride secretion by Vp-TDH, however, is observed in Caco-2 and IEC-6 cells, although calcium-independent

cell death was observed at a high concentration of the toxin (Raimondi *et al*., 2000). Vp-TDH increases the intracellular calcium level and modulates the cytoskeletal organization of IEC-6 cells (Fabbri *et al*., 1999).

A number of reports have described the structural-functional relationship of Vp-TDH (Tang *et al*., 1994; Honda *et al*., 1989; Toda *et al*., 1991; Baba *et al*., 1992; Iida *et al*., 1995). Tang *et al*. (1994) reported the isolation of an interesting mutant toxin of Vp-TDH in which the hemolytic activity had diminished almost completely, but retained the ability to bind to erythrocytes. The mutant toxin, R7, possessed an amino acid substitution at Gly 62 to Ser 62, suggesting that the site is functionally important for Vp-TDH. R7 has been used as a probe to identify the receptor for Vp-TDH (Tang *et al*., 1997a). Tang *et al*. (1997b) analyzed the functional domains of TDH and demonstrated that the N-terminal region may be involved in the binding process while the region near the C-terminal may be involved in the postbinding process. Lang *et al*. (2004) showed that Vp-TDH induces the cation permeability and the breakdown of phosphatidylserine asymmetry of the membrane, a typical feature of erythrocyte apoptosis. Apoptosis by Vp-TDH was also demonstrated by Naim *et al*. (2001).

Vp-TDH shows various biological activities, including lethal toxicity, cytolytic activity (Sakurai *et al*., 1976), and cardiotoxicity (Honda *et al*., 1976a), in addition to the enterotoxicity. Honda *et al*. (1976b) purified the lethal toxin from culture filtrate of *V. parahemolyticus* and found that it was identical to Vp-TDH. Intravenous injection of 5 μg of purified Vp-TDH killed mice within 1 minute and even 1 μg of the toxin killed mice within 20 minutes (Honda *et al*. 1976b). Honda *et al*. (1976b) showed that the lethal activity was due to its cardiotoxic activity. An electrocardiogram of rats injected with TDH showed a wider and higher P wave, suggesting changes in the conduction of an intra-atrial impulse, and an increase in voltage of QRS, suggesting changes in intra-ventricular impulses of electrical activation. Thereafter, the PQ intervals became longer, suggesting inhibition of atrio-ventricular conduction. The cardiotoxicity of Vp-TDH was also demonstrated using cultured mouse heart cells (Honda *et al*., 1976a).

KP-negative strains are occasionally suspected to be the causative agent of food poisoning. Some of those strains produce a TDH-related hemolysin called TRH, which is not detected by Wagatsuma's agar test (Honda *et al*., 1988; Honda *et al*., 1989; Honda *et al*., 1990). TRH shows enterotoxicity and has common antigenicity with TDH, but is thermolabile. Kelly and Stroh (1988) reported that clinical isolates obtained from patients with locally acquired gastroenteritis in Canada all hydrolyzed urea, but none of the isolates was KP-positive, suggesting that the urease-positive strains are the predominant biotype of *V. parahemolyticus* associated with gastroenteritis in the Pacific Northwest. However, Oosawa *et al*. (1996) reported evidence suggesting that urea hydrolysis is not a reliable marker for identifying *tdh*-carrying *V. parahemolyticus* strains in Japan, but may be a marker for *trh*-carrying strains. Okuda *et al*. (1997) analyzed the *tdh* gene and *trh* genes in urease-positive ($Ure^+$) strains of *V. parahemolyticus* isolated on the west coast of the United States and indicated a very strong correlation between the $Ure^+$ phenotype and the *trh*, gene reported for strains isolated in Asia (Suthienkul *et al*., 1995). Iida *et al*. (1997) showed genetic linkage of the *ure* and *trh* genes. Analysis with pulsed field gel electrophoresis of *NotI*-digested DNA fragment and accurate polymerase chain reaction showed localization of *tdh, trh* and *ure* within 40 kb and close proximity of *trh* and *ure* at less than 8.5 kb (Iida *et al*., 1998). The urease gene cluster, however, is not involved in the regulation of *tdh* and *trh* expression (Park *et al*., 2000; Nakaguchi *et al*., 2003).

A TDH gene (*tdh*) having 567 bp was cloned and the DNA sequence was determined (Kaper *et al*., 1984; Taniguchi *et al*., 1985). Nine *tdh* genes have been demonstrated and *tdh2* is thought actually to express in the KP positive strain (Nishibuchi and Kaper, 1990; Nishibuchi and Kaper, 1995). The *tdh* genes are usually, but not exclusively, located in the chromosome. All of the cloned *tdh* genes encode predicted protein products composed of 189 amino acid residues (including signal peptides), which have hemolytic and other biological activities. The nucleotide sequences of the various *tdh* genes are well conserved (greater than 97% identity), and the protein products are immunologically indistinguishable. Similarity of *tdh2* with *trh1* is 68.6%. Similar genes to *tdh* are also found in *V. cholerae* non-O1, *Vibrio mimicus*, and *Vibrio hollisae* (Nishibuchi and Kaper, 1990). These vibrios produce hemolysin similar to Vp-TDH; the hemolysins from the former two are thermostable, but the hemolysin from the latter is thermolabile (Table 44.2). *V. parahemolyticus* possesses two circled chromosomes (Tagomori *et al*., 2002). The complete genome sequence of an epidemic strain of *V. parahemolyticus* RIMD2210633 was published and revealed the presence of a pathogenicity island having *tdh* genes on chromosome 2 (Makino *et al*., 2003).

In addition to TDH and TRH, production of two hemolysins, LDH (lecithin dependent hemolysin) and δ-VPH, by *V. parahemolyticus* was reported. Taniguchi *et al*. (1985) obtained a transformant producing LDH, which was designated as a thermolabile hemolysin (TLH) in their paper. Because of the universal existence

of *tlh* encoding LDH/TLH, the gene is expected to be a tool for the identification of *V. parahemolyticus* (Bej *et al*., 1990). Although LDH of *V. parahemolyticus* had been reported to be a phospholipase A2, Shinoda *et al*. (1991) demonstrated that it should be classified as a phospholipase B or an atypical phospholipase to be designated as phospholipase A2/lysophospholipase. Taniguchi *et al*. (1990) cloned a gene encoding another hemolysin, δ–VPH. The production of δ–VPH was observed in *Escherichia coli* transformed by the cloned gene, but not in *V. parahemolyticus* culture, although all strains of the vibrio possessed its gene. Contribution of these two hemolysins to the pathogenicity has not been demonstrated.

## *VIBRIO VULNIFICUS* HEMOLYSINS

The first isolation of *V. vulnificus* was from a leg ulcer, although it was reported as a *V. parahemolyticus* infection (Roland, 1970). Although the bacterium showed similar characteristics to *V. parahemolyticus*, including having a slightly halophilic property and being negative in sucrose fermentation, it was different in that it had positive lactose fermentation (Hollis *et al*., 1976). The bacterium was therefore called lactose-positive vibrio (Hollis *et al*., 1976), and was subsequently termed *V. vulnificus* (Farmer, 1979). The bacterium causes two types of illness, primary septicemia and wound infection (Blake *et al*., 1980). The former is remarkable for its high fatality rate. *V. vulnificus* is now recognized as being among the most rapidly fatal of human pathogens. In the majority of cases, primary septicemia is associated with the consumption of raw seafood, especially shellfish such as oysters, contaminated with the vibrio. In the United States, 95% of all seafood-related deaths are due to *V. vulnificus*, most commonly from the consumption of raw oysters (Klontz *et al*., 1988; Oliver, 1995). Primary septicemia due to *V. vulnificus* is an opportunistic infection, although most patients with septicemia have underlying disease(s) of liver dysfunction, alcoholic cirrhosis, or hemochromatosis, which leads to an increased plasma iron level and decreased host defense system. Infections in patients having malignant tumors or Acquired Immunodeficiency Syndrome have also been reported. In two-thirds of patients, edematous and/or hemorrhagic secondary skin lesions appear on the extremities and the trunk. Symptoms of the digestive tract, such as diarrhea or vomiting, are very rare. Wound infection is characterized by the development of edema, erythema, or necrosis around a new wound exposed to seawater. This type of infection can occur in healthy persons, as well as in compromised hosts, and may occasionally progress to septicemia.

Tison *et al*. (1982) documented that *V. vulnificus* strains isolated from diseased eels differed phenotypically from typical human isolates. They classified these strains as *V. vulnificus* biotype 2. This biotype is primarily an eel pathogen, and it rarely causes human infections.

*V. vulnificus* produces various exocellular toxic factors, such as hemolysin or protease (Miyoshi *et al*., 1993; Miyoshi and Shinoda, 1997). This vibrio protease is a metalloprotease and degrades a number of biologically important proteins, including elastin, fibrinogen, and plasma proteinase inhibitors of complement components (Miyoshi *et al*., 1995). The protease is also known to enhance vascular permeability through activation of the Factor XII-plasma kallikrein-kinin cascade (Miyoshi *et al*., 1993) and/or exocytotic histamine release from mast cells (Miyoshi *et al*., 2003a), and to form a hemorrhagic lesion through digestion of the vascular basement membrane, which finally provokes severe dermonecrotis (Miyoshi *et al*., 1998). Thus, the protease is the most probable candidate for tissue damage and bacterial invasion during an infection. *V. vulnificus* protease is further thought to supply iron, an essential element for bacterial growth, through cooperation with hemolysin; the hemolysin releases hemoglobin from erythrocytes and the protease liberates iron (protoheme) from the hemoglobin (Nishina *et al*., 1992).

Gray and Kreger (1985) purified a heat-labile hemolysin from a clinical isolate of *V. vulnificus* by ammonium sulfate precipitation, gel filtration with Sephadex G-75, hydrophobic interaction chromatography with Phenyl-Sepharose CL-4B, and isoelectric focusing. This hemolysin, which is currently called VVH (*V. vulnificus* hemolysin) or vulnificolysin, has an isoelectric point of 7.1 and is a hydrophobic 56 kDa single chain polypeptide. Oh *et al*. (1993) found that, in the case of a biotype 2 strain, CHAPS [(3-cholamidopropyl) dimethylammonio-1-panesulfinate] treatment of the preparation obtained by ammonium sulfate precipitation was more efficient for purification of the hemolysin.

The DNA fragment encoding the hemolysin gene was cloned and sequenced (Yamamoto *et al*., 1990b); the sequence contained two open reading frames, *vvhA* and *vvhB*. The *vvhA* is a structural gene for the hemolysin, and encodes a 50,851 Da polypeptide preceded by a 20-amino acid signal peptide. The *vvhB* gene encodes an 18,082 Da polypeptide, but the function of this polypeptide has not been determined. The fragment of the *vvhA* gene has been used as a DNA probe for identification of the vibrio, because this fragment showed 100% specificity and sensitivity for

*V. vulnificus* strains isolated from both clinical and environmental sources. Furthermore, Brauns *et al*. (1991) succeeded in detecting viable but nonculturable *V. vulnificus* cells using the polymerase chain reaction based on amplification of the *vvhA* gene.

Purified VVH is active against erythrocytes from many animal species and against various cultured cells, such as CHO, HeLa, or pulmonary artery endothelial cells. Mouse, sheep, pig, monkey, burro, cat, and pigeon erythrocytes are more sensitive to the hemolysin, while those from rabbit, human, and chicken are less sensitive (Yamanaka *et al*., 1989). In addition, the sublytic doses of the hemolysin can trigger the apoptotic signaling pathway in human ECV304 cells, a vascular endothelial cell strain (Kwon *et al*., 2001).

The hemolytic process has been studied using sheep or mouse erythrocytes as target cells (Shinoda *et al*., 1985; Yamanaka *et al*., 1987b; Kim *et al*., 1993). Hemolysis induced by VVH is temperature-dependent and is optimal between 30 and 37°C. At 4°C, although no erythrocytes are disrupted, the hemolysin can bind to them irreversibly. Therefore, hemolysis by VVH is believed to be a two-step process consisting of a temperature-independent, toxin-binding step followed by a temperature-dependent cell disruption step. Shinoda *et al*. (1985) reported that a small amount of cholesterol abolished the hemolytic ability of VVH, whereas other lipid components in the erythrocyte membrane had no effect on the activity. Yamanaka *et al*. (1987a) found that VVH was active against cholesterol-phosphatidylcholine liposome, but liposome containing a negligible amount of cholesterol was far less sensitive to the action of the hemolysin. No inhibitory effect of cholesterol was recognized when it was added after the hemolysin had bound to the erythrocytes or liposomes. These findings strongly suggest that cholesterol is the binding site for VVH.

At 37°C, VVH treatment of erythrocytes induced rapid $K^+$ efflux that was followed by the release of hemoglobin (Yamanaka *et al*., 1987b). This phenomenon indicates that the temperature-dependent cell disruption step can be further divided into two stages: a small-pore-forming stage causing $K^+$ efflux, and a membrane-bursting stage eliciting the release of hemoglobin (i.e., hemolysis). Namely, intracellular $K^+$ initially pass through the small transmembrane pore formed by VVH, then extracellular low-molecular-weight substances and water enter the erythrocyte via the pore, resulting in the physical explosion of erythrocyte membranes owing to the increased intracellular osmotic pressure. The initial pore-forming stage is temperature-dependent, while the latter one is temperature-independent. Hemoglobin release, but not $K^+$ efflux, from VVH-treated erythrocytes was inhibited completely by the addition of 30 mM (equivalent to intracellular hemoglobin) dextran 4 (molecular diameter of 3.5 nm) and partially by inulin (2.8 nm), although raffinose (1.2 nm) had no effect, denoting that the diameter of the transmembrane pore formed by VVH may be around 3 nm (Yamanaka *et al*., 1987b). The hemolytic reaction by VVH was also inhibited by the addition of a divalent cation such as $Ca^{2+}$, $Mg^{2+}$, or $Mn^{2+}$ (Shinoda *et al*., 1985). However, neither the toxin-binding nor the small pore-forming process interfered with the cation (Yamanaka *et al*., 1987b). Park *et al*. (1994) found that, as well as dextran 4 and other oligosaccharides, calcium functioned as an osmotic protectant preventing the final membrane-bursting stage.

Gray and Kreger (1985) and Shinoda *et al*. (1985) reported that more than one VVH molecule might be required to lyse a single erythrocyte. This suggestion was subsequently supported by experiments using liposome (Yamanaka *et al*., 1987a). When VVH was allowed to act on the cholesterol-phosphatidylcholine liposome entrapping $K^+$, the leakage of $K^+$ accompanied by the formation of the hemolysin oligomer with a molecular weight of about 200 kDa was observed, suggesting that some VVH molecules assembled and formed a single pore on a target cell membrane. The oligomerization of the hemolysin molecules also goes on the erythrocyte membrane (Kim *et al*., 1993) and cholesterol particles (Miyoshi *et al*., 2004). The hemolytic reaction induced by a hemolysin from enteropathogenic *Vibrio* species continues for a long period. Thus, all erythrocytes in the assay tube are disrupted by prolonged incubation (Miyoshi *et al*., 1997b). By contrast, the hemolysis by VVH terminates within a few hours, and the rate of the hemolysis depends upon the amount of the hemolysin.

During cultivation of the vibrio, the hemolytic activity in the culture supernatant reaches a maximum at logarithmic growth phase; however, at early stationary phase, the activity decreases drastically, inversely proportional to the secretion of the protease. Shao and Hor (2000) found that the extracellular hemolytic activity of a protease-deficient mutant, which was constructed by *in vivo* allelic exchange, was about twice that of the parental strain and was retained into the stationary growth phase, indicating proteolytic inactivation of VVH. Indeed, purified *V. vulnificus* protease could convert the hemolysin to the nicked protein through cleavage into 40 kDa and 10 kDa fragments joined together with disulfide bond(s) (Miyoshi *et al*., 2004). In the case of hemolysin from a biotype 2 strain, which is gradually inactivated by autoaggregation because of a highly hydrophobic property, it was modified to a

more hydrophilic and stable form through nicking by the protease (Miyoshi *et al*., 1997a).

Purified VVH has been described as possessing the vascular permeability-enhancing activity. This permeability-enhancing reaction is abolished by the simultaneous administration of an antihistaminic agent. Yamanaka *et al*. (1990) and Kim *et al*. (1998) reported that the hemolysin elicited the histamine liberation from isolated mast cells, and that this liberation was accompanied by the leakage of lactate dehydrogenase, indicating the lysis of mast cells. Thus, VVH inoculated into the dorsal skin may act directly on and disrupt mast cells, resulting in the leakage of histamine and enhancement of the vascular permeability. Gray and Kreger (1987) stated that VVH injected subcutaneously into mice caused severe structural alteration of the skin, which was very similar to the skin lesion shown in *V. vulnificus* infection. In addition, the enzyme-linked immunosorbent assay using the polyclonal or monoclonal antibody demonstrated *in vivo* VVH production by the bacterium (Gray and Kreger, 1989).

Gray and Kreger (1985) have reported that purified VVH administered by the intravenous route was lethal for mice (the 50% lethal dose was about 3.0 μg/kg). This hemolysin has also been documented to cause severe hypotension and vasodilatation through induction of expression of the nitric oxide synthase gene (Kang *et al*., 2002) or activation of guanylate cyclase (Kook *et al*., 1996). These findings suggest that VVH might contribute to the development of systemic *V. vulnificus* infection. However, Morris *et al*. (1987) demonstrated that the virulent potential of individual strains in mice did not correlate with *in vitro* ability to elaborate VVH. Furthermore, Massad *et al*. (1988) and Wright and Morris (1991) reported that hemolysin-negative mutants were as virulent as wild-type strains in mouse models. Thus, VVH may be less important in the development of systemic *V. vulnificus* infection, although it is clear that it plays some role in the pathogenesis.

Testa *et al*. (1984) found that *V. vulnificus* produced phospholipase(s). The partially purified preparation could hydrolyze both acyl ester bonds of all classes of phospholipids except sphingomyelin, demonstrating that the phospholipase preparation possesses both phospholipase A2 and lisophospholipase activities. Therefore, as LDH from *V. parahemolyticus* (Shinoda *et al*., 1991), *V. vulnificus* phospholipase may elicit hemolysis in the presence of lecithin. A novel hemolysin gene *vllY* has been cloned and sequenced by Chang *et al*. (1997). The product of this gene has a molecular weight of 40 kDa and an isoelectric point of 7.1, and its deduced amino acid sequence is similar to legiolysin, a hemolytic toxin from *Legionella pneumophila*.

## *VIBRIO MIMICUS* HEMOLYSINS

*V. mimicus*, a species closely related to *V. cholerae* (Davis *et al*., 1981), is a causative agent of human gastroenteritis. Pathogenic strains of *V. mimicus* show various clinical symptoms, from watery to dysentery-like diarrhea (Hoge *et al*., 1989), suggesting that this pathogen produces many kinds of virulent factors. Enterotoxins similar to CT (Chowdhury *et al*., 1987; Spira and Fedorka-Cray, 1984) and heat-stable enterotoxin (Gyobu *et al*., 1988) have been found in some clinical strains to be causative factors of watery diarrhea. However, most virulent strains lack the ability to produce any of these enterotoxins; in such cases, enterotoxic hemolysins are thought to be candidates for the enteropathogenic factors. Honda *et al*. (1987) found production of two types of hemolysin by *V. mimicus*: One was heat-labile and immunologically similar to VCC (El Tor hemolysin), while another was heat-stable and closely related to Vp-TDH.

The heat-stable hemolysin called Vm-rTDH was purified and characterized by Yoshida *et al*. (1991), and Terai *et al*. (1991) determined the nucleotide sequence of the structural gene of the hemolysin (*tdh*). Shinoda *et al*. (2004) documented that about 30% of the clinical isolates had the *tdh* gene, while this gene was not present in any of the environmental isolates.

Shinoda *et al*. (1993) studied the hemolytic mechanism of the heat-labile hemolysin designated *V. mimicus* hemolysin (VMH). The VMH was indicated to be a member of the pore-forming hemolysins and thought to form a transmembrane pore with a diameter of about 3 nm. The pore formed by the hemolysin was permeable by water and monovalent ions, such as $Na^+$ and $K^+$, but impermeable by hemoglobin. This suggests that VMH causes hemolysis in a colloid osmotic manner. Miyoshi *et al*. (1997b) purified VMH, of which molecular weight is 63 kDa, by ammonium sulfate precipitation followed by column chromatography on Phenyl Sepharose HP and Superose 6 HR. The hemolytic reaction induced by VMH continued up to disruption of all erythrocytes in the assay system. Moreover, VMH bound preliminarily to erythrocyte ghosts showed sufficient ability to attack intact erythrocytes. These results suggest reversible binding of the hemolysin molecule to the membrane. The final cell disruption stage was effectively inhibited by various divalent cations, and some, such as $Zn^{2+}$ and $Cu^{2+}$, blocked the pore-forming stage at higher concentration. Although VMH could disrupt various mammalian erythrocytes including bovine, rabbit, sheep, human, and mouse, those from horse were most sensitive to the hemolysin. Horse erythrocyte was found to

have the most toxin-binding sites and to be hemolyzed by the least membrane-bound toxin molecules, suggesting that toxin-binding to and pore formation on horse erythrocyte takes place most effectively.

Purified VMH induced fluid accumulation into a ligated rabbit ileal loop in a dose-dependent manner, and the antibody against the hemolysin obviously reduced enteropathogenicity of living *V. mimicus* cells (Miyoshi *et al*., 1997b). These findings clearly demonstrate that VMH relates to virulence of this human pathogen. The C-terminal region of VMH was suggested to be essential for induction of the fluid accumulation. Namely, the 50 kDa derivative obtained through proteolytic removal of the C-terminal 13 kDa polypeptide caused negligible fluid accumulation (Miyoshi *et al*., 2003b).

The structural gene of VMH (*vmhA*) was cloned and the nucleotide sequence was determined (Rahman *et al*., 1997; Kim *et al*., 1997). A 2,232 bp open reading frame encodes a polypeptide of 744 amino acid residues, and has a calculated molecular weight of 83,903 Da. The sequence of the gene was closely related to the VCC gene shown above, with 76% homology (Figure 44.1). The 13 amino acid residues of the amino terminus of 63 kDa mature VMH were identical from S-152 to T-164, as predicted from the nucleotide sequence. So, it seems that the mature VMH is processed upon deleting the first 151 amino acids, and the molecular weight is 65,972 Da. Analysis of the deduced amino acid sequence showed the existence of a potential signal sequence of 24 amino acids at the amino terminal, suggesting that, like VCC, two-step processing also exists in VMH maturation. Since all *V. mimicus* strains tested were revealed to have the *vmhA* gene (Shinoda *et al*., 2004), this toxin gene may be a suitable target for genetic identification of *V. mimicus*.

The phospholipase A gene (*phl*) was identified by Kang *et al*. (1998), and Lee *et al*. (2002) isolated its gene product (PhlA) from a transformant. The recombinant PhlA isolated showed phospholipase A activity, but not lisophospholipase, sphingomyelinase, or phospholipase C activity, and also elicited cytolysis of several fish erythrocytes and cultured cells.

## OTHER *VIBRIO* HEMOLYSINS

In addition to the above, some vibrio species have been reported to be pathogenic to human or fish. *Vibrio fluvialis, Vibrio furnissii*, and *Vibrio hollisae* cause gastroenteritis to human, whereas *Vibrio damsella* is a pathogen of extraintestinal infection. Extraintestinal infections by *Vibrio alginolyticus* and *Vibrio metscnikovii* have also been reported occasionally (Blake *et al*., 1980). These vibrios pathogenic to humans inhabit a brackish or seawater environment; *Vibrio anguillarum* is found in fresh or brackish water and is a fish pathogen.

These vibrios also produce hemolysins. *Vibrio fluvialis* is believed to be a bacterium that is pathogenic to humans, in that it is implicated in food poisoning. The infection is usually associated with the consumption of seafood. Kothary *et al*. (2003) purified a heat-labile hemolysin by hydrophobic interaction chromatography with Phenyl-Sepharose CL-4B and gel filtration with Sephacryl S-200. The purified hemolysin has a molecular weight of 63 kDa and an isoelectric point of 4.6, and shows the hemolytic activity against various kinds of erythrocytes and the enterotoxic activity in the suckling mice model. This hemolysin is immunologically and genetically related to VCC. *V. furnissii* is a new species distinguished from *V. fluvialis* by its property of gas production, and is thought to cause diarrhea in humans, although this has not been well established due to the small number of clinical cases. It has been reported to produce a hemolysin that is immunologically indistinguishable from that of *V. fluvialis* (Yamada *et al*., 1988).

*V. hollisae* sometimes causes gastroenteritis with symptoms including diarrhea, abdominal pain, and fever; in rare cases, it causes bacteremia. A hemolysin (Vh-rTDH) is thought to be the diarrheagenic factor. Vh-rTDH resembles Vp-TDH, is 86% homologous in amino acid sequences, but is heat-labile (Yoh *et al*., 1989).

A hemolysin gene of *V. anguillarum* was cloned (Hirono *et al*., 1996). The open reading frame of the gene was 2,253 bp and corresponded to a protein of 751 amino acid residues. The deduced amino acid sequence showed a significant degree of homology with that of VCC and VVH, and the overall amino acid identities were 57.3% and 25.5%, respectively. However, contribution to pathogenicity of this hemolysin has not been documented. *Vibrio tubiashi*, a pathogen of bivalve mollusks, produces a heat-labile hemolysin similar to VVH (Kothary *et al*., 2001). Like VVH, the hemolysin purified was found to be sensitive to cholesterol and to cause hemolysis by a multihit process that is dependent on temperature.

Although *V. metschnikovii* was recognized more than 100 years ago, there have been few reports of isolation of this organism as a human pathogen. It has occasionally been implicated in bacteremia, cholecystitis, diarrhea, and urinary tract infections. A hemolysin produced by this bacterium has been purified and characterized as a pore-forming toxin (Miyake *et al*., 1988; Miyake *et al*., 1989).

*V. damsela* has been reported to cause wound infection. It may cause localized or wide-ranging cellulitis and sometimes more severe complications, such as

disseminated intravascular coagulation. A hemolysin (damselysin) was purified and its phospholipase D activity was reported (Kreger *et al*., 1987). The gene encoding damselysin was cloned and sequenced, but no homology was observed with the sequence of other vibrio hemolysins (Cutter and Kreger, 1990).

## CONCLUSION

Hemolysins produced by pathogenic vibrio are classified into three groups, namely the VCC group, TDH group, and others. This review focused on four hemolysins: VCC (*V. cholerae*), Vp-TDH (*V. parahemolyticus*), VVH (*V. vulnificus*), and VMH (*V. mimicus*), which have been extensively studied. All four are pore-forming toxins, but their precise mechanism of action remains to be investigated. VCC, Vp-TDH, and VMH have activity that causes diarrhea, the major symptom of infection of these vibrios, although VCC is no more than a supplemental factor of CT and VMH is only one of the many enterotoxic factors. Vp-TDH is thought to be the major pathogenic factor of *V. parahemolyticus*, which is one of the most important food poisoning bacterium in Japan and other eastern and southeast Asian countries; the mechanism causing diarrhea, however, has not been identified. Furthermore, it is unclear whether a similar mechanism with the pore-forming action on the erythrocytes contributes to cause diarrhea. Molecular biological studies of the toxins, such as nucleotide and amino sequence, functional domain, or mode of secretion from cell and processing, have been well developed in the past one or two decades. Total genome analysis of *V. cholerae, V. parahemolyticus*, and *V. vulnificus* has been completed in the past half-decade. This genetic information will stimulate the progress of studies by elucidating the pathogenic mechanism for prevention of the disease.

## REFERENCES


Albert, M.J., Ansaruzzaman, M., Bardhan, P.K., Faruque, A.S.G., Faruque, S.M., Islam, M.S., Mahalanabis, D., Sack, R.B., Salam, M.A., Siddique, A.K., Yunus, M.D. and Zaman, K. (1993). Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. *Lancet* **342,** 387–390.

Baba, K., Shirai, H., Terai, A., Kumagai, K., Takeda, Y. and Nishibuchi, M. (1991). Similarity of the *tdh* gene-bearing plasmids of *Vibrio cholerae* non-O1 and *Vibrio parahaemolyticus. Microb. Pathog*. **10,** 61–70.

Bej, A. K., Patterson, D.P., Brsher, C.W., Vicky, M.C.L., Jones, D.D. and Kaysner, C.A. (1990). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shell fish using multiplex PCR amplification of *tl, tdh, and trh. J. Microbiol. Meth*. **36,** 215–225.

Blake, P.A., Weaver, R.E. and Hollis, D.G. (1980). Disease of humans other than cholera) caused by vibrios. *Ann. Rev. Microbiol*. **34,** 341–367.

Brauns, L.A., Hudson, M.C. and Oliver, J.D. (1991). Use of polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl. Env. Microbiol*. **57,** 2651–2655.

Chakraborty, S., Nair, G.B. and Shinoda, S. (1997). Pathogenic vibrios in the natural aquatic environment. *Rev. Environ. Health* **12,** 63–80.

Chang, T.M., Chuang, Y.C., Su, J.H. and Chang, M.C. (1997). Cloning and sequence analysis of a novel hemolysin gene (*vllY*) from *Vibrio vulnificus. Appl. Environ. Microbiol*. **63,** 3851–3857.

Chowdhury, M.A.R., Aziz, K.M.S., Kay, B.A. and Rahim, Z. (1987). Toxin production by *Vibrio mimicus* strains isolated from human and environmental sources in Bangladesh. *J. Clin. Microbiol*. **25,** 2200–2203.

Coelho, A., Andrade, J.R.C., Vincente, A.C.P. and DiRita, V. (2000). Cytotoxic cell vacuolating activity from *Vibrio cholerae* hemolysin. *Infect. Immun*. **68,** 1700–1705.

Cutter, D.L. and Kreger, A.S.(1990). Cloning and expression of the damselysin gene from *Vibrio damsela. Infect. Immun*. **58,** 266–268.

Davis, B.R., Fanning, G.R., Madden, J.M., Steigerwalt, A.G., Bradford, H.B. Jr., Smith, H.L. and Brenner, D.J. (1981). Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus. J. Clin. Microbiol*. **14,** 631–639.

Fabbri, A., Falzano, L., Frank, C., Donelli, G., Matarrese, P., Raimondi, F. Fasano, A. and Fiorentini, C. (1999). *Vibrio parahaemolyticus* thermostable direct hemolysin modulates cytoskeletal organization and calcium homeostasis in intestinal cultured cells. *Infect. Immun*. **67,** 1139–1148.

Fallarino, A., Attridge, S.R., Manning, P.A. and Focareta, T. (2002). Cloning and characterization of a novel hemolysin in *Vibrio cholerae* O1 that does not directly contribute to the virulence of the organism. *Microbiology* **148,** 2181–2189.

Farmer, J. J. (1979). *Vibrio* (*Beneckea*) *vulnificus*: the bacterium associated with sepsis, septicemia, and the sea. *Lancet* **ii,** 903.

Fasano, A., Baudry, B., Pumplin, D. W., Wasserman, S. S., Tall, B. D. Kelly, J. M. and Kaper, J. B. (1991). *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc. Natl. Acad. Sci. USA* **86,** 5242–5246.

Figueroa-Arredondo, P., Heuser, J.E., Akopyants, N.S., Morisaki, J.H., Giono-Cerezo, S., Enriquez-Rincon, F. and Berg, D.E. (2001). Cell vacuolation caused by *Vibrio chelerae* hemolysin. *Infect. Immun*. **69,** 1613–1624.

Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Fukai, K., Mukai, T. and Ueho, T. (1953). On the bacteriological examination of shirasu-food poisoning. *Med. J. Osaka Univ*. **4,** 299–304.

Gray, L.D. and Kreger, A.S. (1985). Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus. Infect. Immun*., **48,** 62–72.

Gray, L.D. and Kreger, A.S. (1987). Mouse skin damage caused by cytolysin from *Vibrio vulnificus* and by *V. vulnificus* infection. *J. Infect. Dis*. **155,** 236–241.

Gray, L.D. and Kreger, A.S. (1989). Detection of *Vibrio vulnificus* cytolysin in *V. vulnificus*-infected mice. *Toxicon* **27,** 459–464.

Gyobu, Y., Kodama, H. and Uetake, H. (1988). Production and partial purification of a fluid-accumulating factor of non-O1 *Vibrio cholerae. Microbiol. Immunol*. **32,** 565–577.

Hall, R.H. and Drasar, B.S. (1990). *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. *Infect. Immun*. **58,** 3375–3379.

Harvey, R. M., Enson, Y., Lewis, M. L., Greenough, W. B., Ally, K. M. and Panno, R. A. (1966). Hemodynamic effects of dehydration and metabolic acidosis in Asiatic cholera. *Trans Assoc. Am. Physiol*. **29,** 177–186.

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D.,

Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonell, L., Utterback, T., Fleishmann, R.D., Nierman, W.C. and White, O. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae. Nature* **406,** 477–483.

Hirono, I., Masuda, T. and Aoki, T. (1996). Cloning and detection of the hemolysin gene of *Vibrio anguillarum. Microb. Pathog*. **21,** 173–182.

Hoge, C.W., Watsky, D., Pealer, R.N,. Libonati, J.P., Israel, E. and Morris, J.G. Jr. (1989). Epidemiology and spectrum of *Vibrio* infections in a Chesapeake Bay community. *J. Infect. Dis*. **160,** 985–993.

Hollis, D.G., Weaver, R.E., Baker, C.N. and Thronsberry, C. (1976). Halophilic *Vibrio* species isolated from blood cultures. *J. Clin. Microbiol*. **3,** 425–431.

Honda, T. Goshima, K., Takeda, Y., Sugino, Y. and Miwatani, T. (1976a). Demonstration of cardiotoxic activity of thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus. Infect. Immun*. **13,** 163–171.

Honda, T., Taga, S., Takeda, T., Hasibuan, M. A., Takeda, Y. and Miwatani, T. (1976b). Identification of lethal toxin with thermostable direct hemolysin produced by *Vibrio parahaemolyticus* and some physicochemical properties of the purified toxin. *Infect. Immun*. **13,** 133–139

Honda, T. and Finkelstein, R. A. (1979). Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor: another toxic substance produced by cholera vibrios. *Infect. Immun*. **26,** 1020–1027.

Honda, T., Narita, I., Yoh, M. and Miwatani, T. (1987). Purification and properties of two hemolysins produced by *Vibrio mimicus. Jpn. J. Bacteriol*. **42,** 201.

Honda, T., Ni, Y. and Miwatani, T. (1988). Characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect. Immun*. **56,** 961–965.

Honda, T., Ni, Y., Hori, S., Takakura, H., Tsunasawa, S., Sakiyama, F. and Miwatani, T. (1989). A mutant hemolysin with lower biological activity produced by a mutant *Vibrio parahaemolyticus. FEMS Microbiol. Lett*. **61,** 95–100.

Honda, T., Ni, Y., Miwatani, T., Adachi, T. and Kim, J. (1992). The thermostable direct hemolysin of *Vibrio parahaemolyticus* is a pore-forming toxin. *Can. J. Microbiol*. **38,** 1175.

Honda, T. and Iida, T. (1993). The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct hemolysin and related hemolysins. *Rev. Med. Microbiol*. **4,** 106–113.

Ichinose, Y., Yamamoto, K., Nakasone, N., Tanabe, M., Takeda, T., Miwatani, T. and Iwanaga, M. (1987). Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae. Infect. Immun*. **55,** 1090–1093.

Iida, T., Suthenkul, O., Park, K.S., Tang, G.Q., Yamamoto, R.K., Ishibashi, M., Yamamoto, K. and Honda, T. (1997). Evidence for genetic linkage between the *ure* and *trh* genes in *Vibrio parahaemolyticus. J. Med. Microbiol*. **46,** 639–645.

Iida, T., Park, K.S., Suthienkul, O., Kozawa, J., Yamaichi, Y., Yamamoto, K. and Honda, T. (1998). Close proximity of the *tdh, trh*, and *ure* genes on the chromosome of *Vibrio parahaemolyticus. Microbiology* **144,** 2517–2523.

Ikigai, H., Akatsuka, A., Tsujiyama, H., Nakae, T. and Shimamura, T. (1996). Mechanism of membrane damage by El Tor hemolysin of *Vibrio cholerae* O1. *Infect. Immun*. **64,** 2968–2973.

Ikigai, H., Ono, T., Iwata, M., Nakae, T. and Shimamura, T. (1997). El Tor hemolysin of *Vibrio cholerae* O1 forms channels in planar lipid bilayer membranes. *FEMS Microbiol. Lett*. **150,** 249–254.

Ikigai, H., Ono, T., Nakae, T.?Otsuru, H. and Shimamura, T. (1999). Two forms of *Vibrio cholerae* O1 El Tor hemolysin derived from identical precursor protein. *Biochim. Biophys. Acta* **1415,** 297–305.

Janda, J. M., Powers, C., Bryant, R. G. and Abbott, S. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev*. **1,** 245–267.

Kang, J.H., Lee, J.H., Park, J.H., Huh, S.K. and Kong, I.S. (1998). Cloning and identification of a phospholipase gene from *Vibrio mimicus. Biochim. Biophys. Acta* **1394,** 85–89.

Kang, M.K., Jhee, E.C., Koo, B.S., Yang, J.Y., Park, B.H., Kim, J.S., Rho, H.W., Kim, H.R. and Park, J.W. (2002). Induction of nitric oxide synthase expression by *Vibrio vulnificus* cytolysin. *Biochem. Biophys. Res. Commun*. **290,** 1090–1095.

Kaper, J.B., Campen, R.K., Seidler, R.J., Baldini, N.M. and Falkow, S. (1984). Cloning of the thermostable direct or Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus. Infect. Immun*. **45,** 290–292.

Kelly, M.T. and Stroh, E.M. (1988). Temporal relationship of *Vibrio parahaemolyticus* in patients and the environment. *J. Clin. Microbiol*. **26,** 1754–1756.

Kim H.R., Rho, H.W., Jeong, M.H., Park, J.W., Kim. J.S., Park, B.H., Kim, U.H. and Park, S.D. (1993). Hemolytic mechanism of cytolysin produced from *V. vulnificus. Life Sci*. **53,** 571–577.

Kim, G.T., Lee, J.Y, Hu, S.H, Yu, J.H. and Kong, I.S. (1997). Nucleotide sequence of the *vmhA* gene encoding hemolysin from *Vibrio mimicus. Biochim. Biophys. Acta* **1360,** 102–104.

Kim, J.S., Chae, M.R., Chang, K., Parh, K.H., Rho, H.W., Park, B.H., Park, J.W. and Kim, H.R. (1998). Cytotoxicity of *Vibrio vulnificus* cytolysin on rat peritoneal mast cells. *Microbiol. Immunol*. **42,** 837–843.

Klontz, K.C., Lieb, S., Schreiber, M., Janowski, H.T., Baldy, L.M. and Gunn, R.A. (1988). Syndromes of *Vibrio vulnificus* infections: clinical and epidemiological features in Florida cases, 1981–1987. *Ann. Intern. Med*. **109,** 318–323.

Kook, H., Lee, S.E., Baik, Y.H., Chung, S.S. and Rhee, J.H. (1996). *Vibrio vulnificus* hemolysin dilates rat thoracic aorta by activating guanylate cyclase. *Life Sci*. **59,** PL41-47.

Kothary, M.H., Delston, R.B., Curtis, S.K., McCardell, B.A. and Tall, B.D. (2001). Purification and characterization of a vulnificolysin-like cytolysin produced by *Vibrio tubiashii. Appl. Environ. Microbiol*. **67,** 3707–3711.

Kothary, M.H., Lowman, H., McCardell, B.A. and Tall, B.D. (2003). Purification and characterization of enterotoxigenic El Tor-like hemolysin produced by *Vibrio fluvialis. Infect. Immun*. **71,** 3213–3220.

Kreger, A.S., Bernheimer, A.W., Etkin, L.A. and Daniel, L.W. (1987). Phospholipase D activity of *Vibrio damsela* cytolysin and its interaction with sheep erythrocytes. *Infect. Immun*. **55,** 3209–3212.

Kwon, K.B., Yang, J.Y., Ryu, D.G., Rho, H.W., Kim, J.S., Park, J.W. and Park, B.H. (2001). *Vibrio vulnificus* cytolysin induces superoxide anion-initiated apoptotic signaling pathway in human ECV304 cells. *J. Biol. Chem*. **276,** 47518–47523.

Lang, P.A., Kaiser, S., Myssina, S., Birka, C., Weinstock, C., Northoff, H., Wieder, T., Lang, F. and Huber, S.M. (2004). Effect of *Vibrio parahaemolyticus* hemolysin on human erythrocytes. *Cell Microbiol*. **6,** 391–400.

Lee, J.H., Ahn, S.H., Kim, S.H., Choi, Y.H., Park, K.J. and Kong, I.S. (2002). Characterization of *Vibrio mimicus* phospholipase A (PhlA). and cytotoxicity on fish cell. *Biochem. Biophys. Res. Commun*. **298,** 269–276.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A., Kubota, Y., Kimura, S., Yasunaga, T., Honda, T., Shinagawa, H., Hattori, M. and Iida T. (2003). Genome sequence of *Vibrio parahaemolyticus*: A pathogenic mechanism distinct from that of *V. cholerae. Lancet* **361,** 743–749.

Massad, G., Simpson, L.M. and Oliver, J.D. (1988). Isolation and characterization of hemolysin mutants of *Vibrio vulnificus. FEMS Microbiol. Lett*. **56,** 295–300.

Menzl, K.,Maier, E., Chakraborty, T. and Benz, R. (1996). HlyA hemolysin of *Vibrio cholerae* O1 biotype E1 Tor. Identification of the hemolytic complex and evidence for the formation of anion-selective, ion-permeable channels. *Eur. J. Biochem*. **240,** 646–654.

Mitra, R., Figueroa, P., Mukhopadhyay, A.K., Shinmada, T., Takeda, Y., Berg, D.E. and Nair, G.B. (2000). Cell vacuolation, a manifestation of the El Tor hemolysin of *Vibrio cholerae. Infect. Immun*. **68,** 1928–1933.

Miwatani, T. and Takeda, Y. (1976). *Vibrio parahaemolyticus. A causative bacterium of food poisoning*. Saikon Publishing Co., Tokyo.

Miyake, M., Honda, T. and Miwatani, T. (1988). Purification and characterization of *Vibrio metschnikovii* cytolysin. *Infect. Immun*. **56,** 954–960.

Miyake, M., Honda, T. and Miwatani, T. (1989). Effects of divalent cations and saccharides on *Vibrio metschnikovii* cytolysin-induced hemolysis of rabbit erythrocytes. *Infect. Immun*. **57,** 158–163.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. and Yamai, S. (1969). *In vitro* hemolytic characteristic *Vibrio parahaemolyticus*: its close relation with human pathogenicity. *J. Bacteriol*. **100,** 1147–1149.

Miyamoto, Y., Obara, Y., Nikkawa, T., Yamai, S., Kato, T., Yamada, Y. and Ohashi, M. (1980). Simplified purification and biophysicochemical characteristics of Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus. Infect. Immun*. **28,** 567–576.

Miyoshi, S., Oh, E.-G., Hirata, K. and Shinoda, S. (1993). Exocellular toxic factors produced by *Vibrio vulnificus. J. Toxicol. Toxin Rev*. **12,** 253–288.

Miyoshi, S. Narukawa, H., Tomochika, K. and Shinoda, S. (1995). Actions of *Vibrio vulnificus* metalloprotease on human plasma proteinase-proteinase inhibitor systems: A comparative study of native protease with its derivative modified by polyethylene glycol. *Micobiol. Immunol*. **39,** 959–966.

Miyoshi, S., Fujii, S., Tomochika, K. and Shinoda, S. (1997a). some properties of nicked *Vibrio vulnificus* hemolysin. *Microbial Pathog*. **23,** 235–239.

Miyoshi, S., Sasahara, K., Akamatsu, S., Rahman, M. M., Katsu, T., Tomochika, K. and Shinoda, S. (1997b). Purification and characterization of a hemolysin produced by *Vibrio mimicus. Infect. Immun*. **65,** 1830–1835.

Miyoshi, S. and Shinoda, S. (1997). Bacterial metalloprotease as the toxic factor in infection. *J. Toxicol. Toxin Rev*. **16,** 177–194.

Miyoshi, S., Nakazawa, H., Kawata, K., Tomochika, K., Tobe, K. and Shinoda, S. (1998). Characterization of the hemorrhagic reaction caused by *Vibrio vulnificus* metalloprotease, a member of the thermolysin family. *Infect. Immun*. **66,** 4851–4855.

Miyoshi, S., Kawata, K., Hosokawa, M., Tomochika, K. and Shinoda, S. (2003a). Histamine-releasing reaction induced by the N-terminal domain of *Vibrio vulnificus* metalloprotease. *Life Sci*. **72,** 2235–2242.

Miyoshi, S., Takata, N., Sakurai, A., Yamamoto, S. and Shinoda, S. (2003b). An enterotoxic hemolysin produced by *Vibrio mimicus. J. Toxicol. Toxin Rev*. **22,** 726–727.

Miyoshi, S., Morita, A., Teranishi, T., Tomochika, K., Yamamoto, S. and Shinoda, S. (2004). An exocellular cytolysin produced by *Vibrio vulnificus* CDC B3547, a clinical isolate in biotype 2 (serovar E). *J. Toxicol. Toxin Rev*. **23,** 111–121.

Morris, J.G. Jr., Wright, A.C., Simpson, L.M., Wood, P.K., Johnson, D.E. and Oliver, J.D. (1987). Virulence of *Vibrio vulnificus*: association with utilization of transferrin-bound iron, and lack of correlation with levels of cytotoxin or protease production. *FEMS Microbiol. Lett*. **40,** 55–59.

Morris, J. G. (1990). Non-O group 1 *Vibrio cholerae*: A look at the epidemiology of an occasional pathogen. *Epidemiol. Rev*. **12,** 179–191.

Nagamune, K., Yamamoto, K. and Honda, T. (1995). Cloning and sequencing of a novel hemolysis gene of *Vibrio cholerae. FEMS Microbiol. Lett*. **128,** 265–269.

Nagamune, K., Yamamoto, K, Naka, A., Matsuyama, J, Miwatani, T. and Honda, T. (1996). *In vitro* proteolytic processing and activation of the recombinant precursor of El Tor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae* by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infect. Immun*. **64,** 4655–4658.

Nagamune, K., Yamamoto, K. and Honda, T. (1997). Intramolecular chaperone activity of the pro-region of *Vibrio cholerae* El Tor cytolysin. *J. Biol. Chem*. **272,** 1338–1343.

Naim, R., Yanagihara, I., Iida, T. and Honda T. (2001). *Vibrio parahaemolyticus* thermostable direct hemolysin can induce an apoptoic cell death in Rat-1 cells from inside and outside of the cells. *FEMS Microbiol. Lett*. **195,** 237–244.

Nishibuchi, M. and Kaper, J.B. (1990). Duplication of the thermostable direct hemolysin (*tdh*) gene in *Vibrio parahaemolyticus. Mol. Microbiol*. **4,** 87–99.

Nishibuchi, M., Fasano, A., Russel, R. G. and Kaper, J. B. (1992). Enterotoxigenicity of *Vibrio parahaemolyticus* with and without genes encoding thermostable direct hemolysin. *Infect. Immun*. **60,** 3539–3545.

Nishibuchi, M. and Kaper, J. B. (1995). Thermostable direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. *Infect. Immun*. **63,** 2093–2099.

Nishina, Y., S. Miyoshi, A. Nagase and S. Shinoda. (1992). Significant role of an exocellular protease in utilization of heme by *Vibrio vulnificus. Infect. Immun*. **60,** 2128–2132.

Oh, E.G., Tamanoi, Y., Toyoda, A., Usui, K., Miyoshi, S., Chang, D.S. and Shinoda, S. (1993). Simple purification method for a *Vibrio vulnificus* hemolysin by a hydrophobic column chromatography. *Microbiol. Immunol*. **37,** 975–978.

Okuda, J., Ishibashi, M., Abbot, S.L., Janda, J.M. and Nishibuchi, M. (1997). Analysis of the thermostable direct hemolysin (t*dh*) gene and the tdh-related hemolysin (*trh*) genes in urease-positive strains of *Vibrio parahaemolyticus* isolated on the West Coast of the United States. *J. Clin. Microbiol*. **35,** 1965–1971.

Oliver, J.D. (1995). The viable but non-culturable state in the human pathogen *Vibrio vulnificus. FEMS Microbiol. Lett*. **133,** 203–208.

Olson, R. and Gouaux, E. (2003). *Vibrio cholerae hemolysin is composed of an a-hemolysin-like core*. Protein Sci. *12, 379–83.*

Oosawa, R., Okitsu, T., Morozumi, H. and Yamai, S. (1996). Occurrence of urease-positive *Vibrio parahaemolyticus* in Kanagawa, Japan, with specific reference to presence of thermostable direct hemolysin (TDH) and the TDH-related-haemolysin genes. *Appl. Environ. Microbiol*. **62,** 725–727.

Pal, S., Guhathakurta, B., Samal, D., Mallick, R. and Datta, A. (1997). Purification and characterization of a hemolysin with phospholipase C activity from *Vibrio cholerae* O139. *FEMS Microbiol. Lett*. **147,** 115–120.

Park, J.W., Jahng, T.A., Rho, H.W., Park B.H., Kim, N.H. and Kim, H.R. (1994). Inhibitory mechanism of $Ca^{2+}$ on the hemolysis caused by *Vibrio vulnificus* cytolysin. *Biochim. Biophys. Acta* **1194,** 166–170.

Park, K.S., Iida, T., Yamaichi, Y., Oyagi, T. Yamamoto, K. and Honda, T. (2000). Genetic characterization of DNA region containing the trh and ure genes of *Vibrio parahaemolyticus. Infect. Immun*. **68,** 5742–5748.

Rahman, M. M., Miyoshi, S., Tomochika, K., Wakae, H. and Shinoda, S. (1997). Analysis of the structural gene encoding a hemolysin in *Vibrio mimicus. Microbiol. Immunol*. **41,** 169–173.

Raimondi, F., Kao, J.P.Y., Fiorentini, C., Fabbri, A., Donelli, G., Gasparini, N., Rubino, A. and Fasano, A. (2000). Enterotoxicity and cytotoxixcity of *Vibrio parahaemolyticus* thermostable direct hemolysin in *in vitro* system. *Infect. Immun*. **68,** 3180–3185.

Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Nair, G. B., Shimada, T., Takeda, T., Kurasawa, T, Kurazono, H., Pal, A. and Takeda, Y. (1993). Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet* **314,** 703–704.

Roland, F.P. (1970). Leg gangrene and endotoxin shock due to *Vibrio parahaemolyticus*. An infection acquired in New England coastal water. *New Engl. J. Med*. **282,**1306.

Saha, N. and Banerjee, K. K. (1997). Carbohydrate-mediated regulation of interaction of *Vibrio cholerae* hemolysin with erythrocyte and phospholipid vesicle. *J. Biol. Chem*. **272,** 162–167.

Sakazaki, R., Iwanami, S. and Fukumi, H. (1968). Studies on the enteropathogenic, facultative halophilic bacteria, *Vibrio parahaemolyticus*. II. Serological characteristics. *Jpn. J. Med. Sci. Biol*. **21,** 313–324.

Sakurai, J., Honda, T., Jinguji, Y., Arita, M. and Miwatani, T. (1976). Cytotoxic effect of the thermostable direct hemolysin produced by *Vibrio parahaemolyticus* on FL cells. *Infect. Immun*. **13,** 876–883.

Shao, C.-P. and Hor, L.-I. (2000). Metalloprotease is not essential for *Vibrio vulnificus* virulence in mice. *Infect. Immun*. **68,** 3569–3573.

Shinoda, S., Miyoshi, S., Yamanaka, H. and Miyoshi-Nakahara, N. (1985). Some properties of *Vibrio vulnificus* hemolysin. *Microbiol. Immunol*. **29,** 583–590.

Shinoda, S., Matsuoka, H., Tsuchie, T., Miyoshi, S., Yamamoto, S., Taniguchi, H. and Mizuguchi, Y. (1991). Purification and characterization of a lecithin-dependent hemolysin from *Escherichia coli* transformed by a *Vibrio parahaemolyticus* gene. *J. Gen. Microbiol*. **137,** 1737–1742.

Shinoda, S., Ishida, K., Oh, E.-G., Sasahara, K., Miyoshi, S., Chowdhury, M.A.R. and Yasuda, T. (1993). Studies on hemolytic action of a hemolysin produced by *Vibrio mimicus. Microbiol. Immunol*. **37,** 405–409.

Shinoda, S., Nakagawa, T., Shi, L., Bi, K., Kanoh, Y., Tomochika, K., Miyoshi, S. and Shimada, T. (2004). Distribution of virulence-associated genes in *Vibrio mimicus* isolates from clinical and environmental origins. *Microbiol. Immunol*. **48,** 547–551.

Spira, W.M. and Fedorka-Cray, P.J. (1984). Purification of enterotoxin from *Vibrio mimicus* that appear to be identical to cholera toxin. *Infect. Immun*. **45,** 679–684.

Suthienkul, O., Ishibashi, M., Iida, T., Nettip, N., Supavej, S., Eampokalap, B., Makino, M. and Honda, T. (1995). Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. *J. Infect. Dis*. **172,** 1405–1408.

Tagomori, K., Iida, T. and Honda, T. (2002). Comparison of genome structures of vibrios, bacteria possessing two chromosomes. *J. Bacteriol*. **184,** 4351–4358.

Takeda, T, Peina Y., Ogawa, a., Dohi, S., Abe, H., Nair, G. B. and Pal, S. C. (1991). Detection of heat-stable enterotoxin in a cholera toxin gene-positive strain of *Vibrio cholerae* O1. *FEMS Microbiol. Lett*. **80,** 23–28.

Tang, G.-Q. Iida, T., Yamamoto, K. and Honda, T. (1994). A mutant toxin of *Vibrio parahaemolyticus* thermostable direct hemolysin, which has lost hemolytic activity but retains ability to bind to erythrocytes. *Infect. Immun*. **62,** 3299–3304.

Tang, G.-Q., Iida, T., Yamamoto, K. and Honda, T. (1995). $Ca^{2+}$-independent cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) on Intestine 407, a cell line derived from human embryonic intestine. *FEMS Microbiol. Lett*. **134,** 233–238.

Tang, G.-Q., Iida, T., Yamamoto, K. and Honda, T. (1995). Ca2+ independent cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) on Intestine 407, a cell line derived from human embryonic intestine. *FEMS Microbiol. Lett*. **134,** 233–238.

Tang, G.-Q., Iida, T., Inoue, H., Yutsudo, M., Yamamoto, K and Honda, T. (1997a). A mutant cell line resistant to *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH): its potential in identification of putative receptor for TDH. *Biochim. Biophys. Acta* **1360,** 277–282.

Tang, G.-Q., Iida, T., Yamamoto, K. and Honda, T. (1997b) Analysis of functional domains of *Vibrio parahaemolyticus* thermostable direct hemolysin using monoclonal antibodies. FEMS *Microbiol. Lett*. **150,** 289–296.

Taniguchi, H., Ohta, H., Ogawa, M. and Mizuguchi, Y. (1985). Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile hemolysin genes. *J. Bacteriol*. **162,** 510–515.

Taniguchi, H., Kubomura, S., Hirano, H., Mizue, K., Ogawa, M. and Mizuguchi, Y. (1990). Cloning and characterization of a gene encoding a new thermostable hemolysin from *Vibrio parahaemolyticus. FEMS Microbiol Lett*. **7,** 339–346.

Terai, A., Shirai, H., Yoshida, O., Takeda, Y. and Nishibuchi, M. (1991). Nucleotide sequence of the thermostable direct hemolysin gene (*tdh* gene) of *Vibrio mimicus* and its evolutionary relationship with *tdh* genes of *Vibrio parahaemolyticus. FEMS Microbiol. Lett*. **59,** 319–323.

Testa, J., L.W. Daniel and A.S. Kreger. (1984). Extracellular phospholipase A2 and lisophospholipase produced by *Vibrio vulnificus. Infect. Immun*. **45,** 458–463.

Tison, D.L., Nishibuchi, M., Greenwood, J.D. and Seidler, R.J. (1982). *Vibrio vulnificus* biotype 2: new biogroup pathogenic for eels. *Appl. Environ. Microbiol*. **44,** 640–646.

Trucksis, M., Galen, J. E., Michalski, J., Fasano, A. and Kaper, J. B. (1993). Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. *Proc. Natl. Acad. Sci. USA*. **90,** 5267–5271.

Tsunasawa, S., Sugihara, A., Masaki, T., Sakiyama, F., Takeda, Y., Miwatani, T. and Narita, K. (1987). Amino acid sequence of thermostable direct hemolysin produced by *Vibrio parahaemolyticus.* J. Biochem. ***101,*** 111–121.

Valeva, A., Walev, I., Weis, S., Boukhallouk, F., Wassenaar, T.M., Endres, K., Fahrenholz, F., Bhakdi, S. and Zittzer, A. (2004). A cellular metalloproteinase activates *Vibrio choleare* pro-cytolysin. *J. Biol. Chem*. **279,** 25143–25148.

Wright, A.C. and Morris, J.G. Jr. (1991). The extracellular cytolysin of *Vibrio vulnificus*: inactivation and relationship to virulence in mice. *Infect. Immun*. **59,** 192–197.

Yamada, S., Matsushita, S., Kudoh, Y. and Ohashi, M. (1988). Purification and characterization of hemolysin produced by *Vibrio fluvialis. Advances in Research on Cholera and Related Diarrheas* **4**: 111

Yamamoto, K., Ichinose, Y., Nakasone, N., Tanabe, M., Nagahama, M. Sakurai, J. and Iwanaga, M. (1986). Identification of hemolysins produced by *Vibrio cholerae* non-O1 and *Vibrio cholerae* O1, biotyoe *eltor. Infect. Immun*. **51,** 927–931.

Yamamoto, K., Ichinose, Y., Shinagawa, H., Makino, K., Nakata, A., Iwanaga, M., Honda, T. and Miwatani, T. (1990a) Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotyoe El Tor: nucleotide sequence of the structural gene (*hly A*) and characterization of the processed products. *Infect. Immun*. **58,** 4106–4116.

Yamamoto, K., Wright, A. C., Kaper, J. B. and Morris, Jr., J. G.(1990b). The cytolysin gene of *Vibrio vulnificus*: sequence and relationship

to the *Vibrio cholerae* El Tor hemolysin gene. *Infect. Immun*. **58,** 2706–2709.

Yamanaka, H., Katsu, T., Satoh, T. and Shinoda, S. (1987a). Effect of *Vibrio vulnificus* hemolysin on liposome membranes. *FEMS Microbiol. Lett*. **44,** 253–258.

Yamanaka, H., Satoh, T., Katsu, T. and Shinoda, S.(1987b) Mechanism of hemolysis by *Vibrio vulnificus* hemolysin. *J. Gen. Microbiol*. **133,** 2859–2864.

Yamanaka, H., Shimatani, S., Tanaka, M., Katsu, T., Ono, B. and Shinoda, S. (1989). Susceptibility of erythrocytes from several animal species to *Vibrio vulnificus* hemolysin. *FEMS Microbiol. Lett*. **61,** 251–256.

Yamanaka, H., Sugiyama, K., Furuta, H., Miyoshi, S. and Shinoda, S. (1990). Cytolytic action of *Vibrio vulnificus* hemolysin on mast cells from rat peritoneal cavity. *J. Med. Microbiol*., **32,** 39–43.

Yoh, M., Honda, T., Miwatani, T., Tsunasawa, S. and Sakiyama, F. (1989). Comparative amino acid sequence analysis of hemolysins produced by *Vibrio hollisae* and *Vibrio parahaemolyticus*. *J. Bacteriol*. **171,** 6859–6861.

Yoh, M., Tang, G.-Q, Iida, T., Morinaga, N., Noda, M. and Honda, T. (1996). Phosphorylation of a 25 kDa protein is induced by thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Int. J. Biochem. Cell Biol*. **28,** 1365–1369.

Yoshida, H., Honda, T. and Miwatani, T. 1991. Purification and characterization of a hemolysin of *Vibrio mimicus* that relates to thermostable direct hemolysin of *Vibrio parahaemolyticus*. *FEMS Microbiol. Lett*. **84,** 249–254.

Zhang, D., Takahashi, J., Seno, T., Tani, Y. and Honda, T. (1999). Analysis of receptor fro *Vibrio cholerae* El Tor Hemolysin with a monoclonal antibody that recognizes glycophorin B of human erythrocyte membrane. *Infect. Immun*. **67,** 5332–5337.

Zhang, D. and Honda, T. (1999). Disappearance of glyceraldehydes 3-phosphate dehydrogenase from erythrocyte membrane by hemolysis with thermostable direct hemolysin of *Vibrio parahaemolyticus* or *Vibrio cholerae* El Tor hemolysin. *Microbiol. Immuno*l. **43,** 303–305.

Zitzer, A., Zitzer, O., Bhakdi, S. and Palmer, M. (1999). Oligomerization of *Vibrio cholerae* cytolysin yields a pentameric pore and has a dual specificity for cholesterol and sphingolipids in the target membrane. J. *Biol. Chem*. **274,** 1375–1380.

CHAPTER

# 45

# *Clostridium perfringens* enterotoxin

*Bruce A. McClane*

## INTRODUCTION

### Enterotoxin-producing *Clostridium perfringens*

The Gram-positive, anaerobic, spore former *C. perfringens* causes an impressive array of enteric and histotoxic diseases in humans and domestic animals. The pathogenic versatility of this bacterium is largely attributable to its prodigious toxin-producing abilities, with at least 14 different *C. perfringens* toxins now reported in the literature.

However, individual *C. perfringens* isolates produce only subsets of this toxin arsenal, forming the basis for a commonly used toxinotype classification scheme (McClane, 2001) that assigns *C. perfringens* isolates to one of five types (A–E) based upon their ability to produce alpha, beta, epsilon, and iota toxins (Table 45.1). About 1–5% of *C. perfringens* isolates, mostly belonging to type A (McClane, 2001), express another biomedically important toxin named *C. perfringens* enterotoxin (CPE). The 35 kDa CPE polypeptide has a unique primary sequence, except for some limited homology (of unknown significance) with the Antp70/C1 protein made by certain strains of *C. botulinum* (Melville *et al*., 1997).

Several interesting features of CPE action, genetics, and expression represent the major focus of this chapter.

### The biomedical importance of enterotoxin-producing *C. perfringens*

Traditionally, CPE-positive type A isolates have been associated with *C. perfringens* type A food poisoning, which ranks among the most common of all foodborne human GI diseases (McClane, 2001). For example, the Centers for Disease Control and Prevention estimate that more than 250,000 cases of this food poisoning occur annually in the U.S.A. (Mead *et al*., 1999).

*C. perfringens* type A food poisoning results from ingestion of foods (usually meat or poultry products) contaminated with high levels of vegetative cells of a CPE-positive type A isolate (McClane, 2001). Those ingested bacteria later sporulate in the intestines, allowing CPE production (further discussion later). Symptoms of *C. perfringens* type A food poisoning typically include diarrhea and abdominal cramps (McClane, 2001), which typically persist for 24 h prior to full recovery; however, this can be a fatal illness in the elderly or debilitated.

Several lines of evidence (McClane, 2001) implicate CPE as the major virulence factor responsible for the symptoms of *C. perfringens* type A food poisoning. For example, CPE is detectable (often at levels known to be enterotoxic in animal models) in the feces of virtually all people suffering from this food poisoning. Additionally, human volunteer feeding studies showed that ingestion of purified CPE is sufficient to reproduce the diarrhea and cramping symptoms of natural *C. perfringens* type A food poisoning. Finally, CPE expression appears to be required for the enteric virulence of most (or all) food poisoning isolates since specific inactivation of the enterotoxin gene (*cpe*) in SM101, a transformable derivative of food poisoning isolate NCTC 8798, produced an isogenic mutant lacking the ability to cause GI effects (e.g., fluid accumulation, histopathologic damage) in rabbit ileal loops (Sarker *et al*., 1999).

In the mid-1980s, enterotoxin-producing type A isolates also became linked to non-foodborne human GI

**TABLE 45.1** *Clostridium perfringens* toxinotypes

| | Toxins Produced | | | |
|---|---|---|---|---|
| **Type** | **Alpha** | **Beta** | **Epsilon** | **Iota** |
| A | + | – | – | – |
| B | + | + | + | – |
| C | + | + | – | – |
| D | + | – | + | – |
| E | + | – | – | + |

diseases (Carman, 1997), including antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD). CPE appears to play an important role in the pathogenesis of many or all AAD/SD cases since specific inactivation of the *cpe* gene in SD isolate F4969 eliminates that isolate's ability to cause enteric pathology in rabbit ileal loops (Sarker *et al*., 1999).

Compared to *C. perfringens* type A food poisoning, the GI symptoms of CPE-associated AAD/SD tend to be more severe and of longer duration (Carman, 1997). Recent studies (Fisher *et al*., 2005) suggest these clinical differences may be attributable, at least in part, to most CPE-positive AAD/SD isolates producing an additional enterically active toxin, i.e., the recently discovered beta2 toxin (Gibert *et al*., 1997). Specifically, Fisher *et al*. found that more than 75% of the CPE-positive AAD/SD isolates they tested could produce beta2 toxin, while only than 15% of the CPE-positive *C. perfringens* food poisoning isolates they examined were able to produce beta2 toxin. Given those results, it is notable that F4969, the CPE-positive SD isolate used for constructing isogenic *cpe* knockout mutants (as described above), was recently determined (Fisher *et al*., 2005) to be an atypical CPE-positive SD diarrhea isolate that is beta2 toxin–negative (see CPE Genetics for further discussion).

CPE-positive isolates have also been linked to certain veterinary GI diseases (Songer, 1996). For example, enterotoxin-producing type A isolates are implicated in canine enteritis (Marks *et al*., 2002). Some type C and D (and perhaps B) veterinary disease isolates also produce CPE, but the contribution (if any) of CPE to the pathogenesis of type C and D veterinary infections has not yet been evaluated.

## THE GENETICS AND EXPRESSION OF CPE

### CPE genetics

#### *The* cpe *gene*

The enterotoxin gene (*cpe*) includes an open reading frame (ORF) encoding a 35 kDa protein of 319 amino acids (Czeczulin *et al*., 1993). The *cpe* ORF appears to be remarkably conserved among type A isolates, i.e., sequencing analyses detected not a single nucleotide difference among the *cpe* ORFs present in eight different type A isolates, whether obtained from food poisoning or AAD/SD sources (Collie *et al*., 1998). Recent observations (Sayeed, Fisher, and McClane, unpublished) indicate that the *cpe* ORF nucleotide sequence present in most (if not all) *cpe*-positive type C and D isolates is identical to the classical *cpe* ORF present in type A human GI disease isolates. Interestingly, most (if not all) type E isolates carry *cpe* ORF sequences that are silent, in part due to the presence of nonsense and frame-shift mutations (Billington *et al*., 1998; and see *cpe* Genotype section, below).

The DNA region immediately upstream of the *cpe* ORF is also reasonably conserved among type A isolates (Melville *et al*., 1994; Zhao and Melville, 1998), except for the presence in some type A isolates of a 45 nucleotide insert about 265 bp upstream of the *cpe* start codon. The presence or absence of that small insert has no apparent effect on CPE expression levels (Collie *et al*., 1998). As shown in Figure 45.1, primer extension and deletion analyses (Zhao and Melville, 1998) demonstrated the presence of at least three promoters directly upstream of the *cpe* ORF (see Regulation of CPE Expression for further discussion).

A putative stem loop structure, followed by an oligo dT tract, is located about 36 bp downstream of the *cpe* ORF in most, if not all, type A isolates (Czeczulin *et al*., 1993). This stem loop–containing region possesses characteristics of a rho-independent transcriptional terminator and may also contribute to *cpe* mRNA stability (see Regulation of CPE Expression for further discussion).

#### *Association of the* cpe *gene with mobile genetic elements*

In type A isolates, the *cpe* gene can be present on either the chromosome or a large plasmid (Cornillot *et al*., 1995; Collie and McClane, 1998); no single type A isolate has yet been found to carry both a chromosomal and plasmid-borne *cpe* gene. The location of the *cpe* gene in type C and D isolates has not yet been determined, but the silent *cpe* sequences present in most/all type E isolates are located on the same plasmid that also carries functional iota toxin genes (Billington *et al*., 1998).

As shown in Fig. 45.2, both the chromosomal and plasmid-borne *cpe* genes of type A isolates are closely associated with insertion sequences (Miyamoto *et al*., 2002; Wen *et al*., 2003; Miyamoto *et al*., 2004). When chromosomal, the *cpe* gene may be present on an about 6.3 kb transposon named Tn*5565* (Brynestad *et al*., 1997; Brynestad and Granum, 1999). This putative

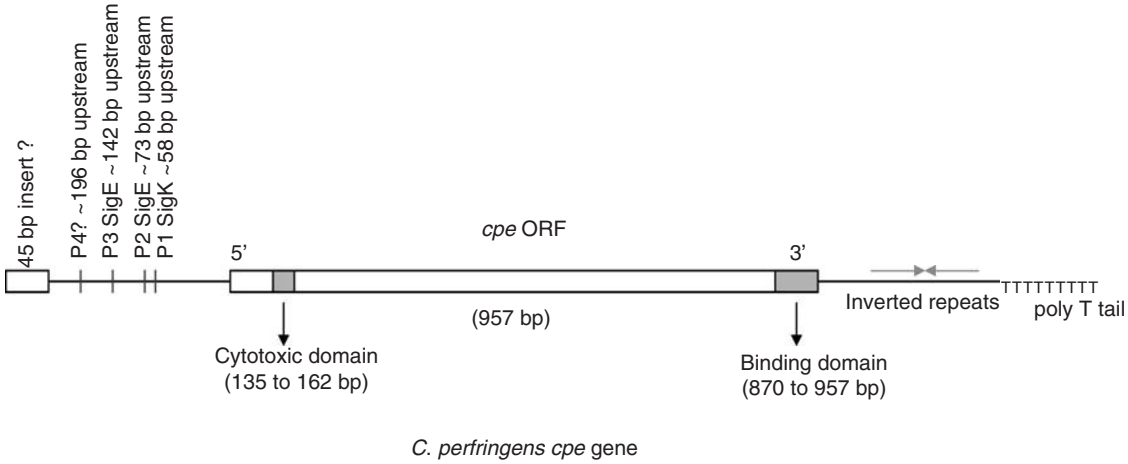



**FIGURE 45.1** Arrangement of the *cpe* gene. Note the presence of at least three promoters (P1, P2, and P3) immediately upstream of the *cpe* ORF as confirmed by primer extension analyses, Rnase T2 protection assays, and deletion mutagenesis (Melville *et al*., 1994; Zhao and Melville, 1998); a fourth potential promoter (P4) identified by primer extension analysis could not be confirmed by other techniques (Melville *et al*., 1994; Zhao and Melville, 1998). A 45 nucleotide insert present upstream of the *cpe* promoters in some type A isolates (Melville *et al*., 1994) has no effect on CPE expression (Collie *et al*., 1998). The 957 nucleotide *cpe* ORF encodes a unique protein of 35 kDa that lacks a signal peptide. Regions of the *cpe* ORF encoding cytotoxic or receptor-binding activity are also indicated in the figure. Finally, a region containing inverted repeats followed by an oligo-T tract is located immediately downstream of the *cpe* termination codon; those sequences may be involved in transcriptional termination and/or message stability (Czeczulin *et al*., 1993).

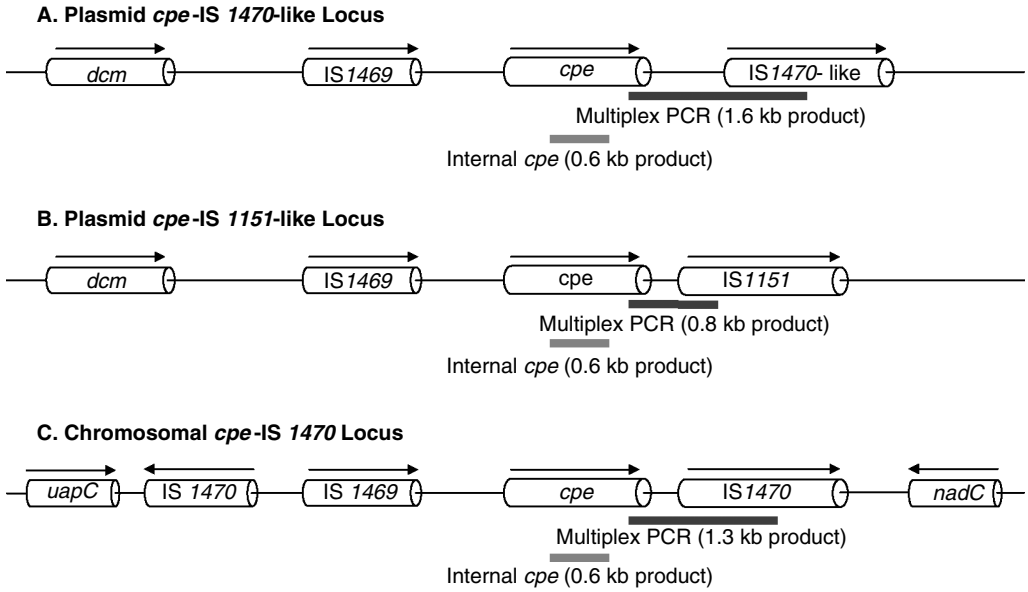



**FIGURE 45.2** Organization of the plasmid *cpe* locus of non-foodborne human GI disease isolates F4969 (A) or F5603 (B) versus the chromosomal *cpe* locus of food poisoning isolate NCTC8239 (C). This figure is based upon results from (Miyamoto *et al*., 2002; Wen *et al*., 2003; Miyamoto *et al*., 2004); those findings were used to develop a multiplex PCR *cpe* genotyping assay (Miyamoto *et al*., 2004) capable of distinguishing between chromosomal *cpe* isolates (C), plasmid *cpe* isolates with IS*1470*-like sequences in their plasmid *cpe* locus (A), and plasmid *cpe* isolates with IS*1151* sequences in their plasmid *cpe* locus (B).

transposon, which carries IS*1469* sequences directly upstream of the *cpe* gene, is flanked on both ends by IS*1470* sequences. Some evidence (Brynestad and Granum, 1999) suggests that Tn*5565* can excise from the chromosome and then form a circular intermediate, which could facilitate *cpe* gene movement. However, actual movement/insertion of Tn*5565* from one DNA location to another has not yet been demonstrated.

As shown in Figure 45.2, IS*1469* sequences are similarly present upstream of the plasmid-borne *cpe* gene in type A isolates (Miyamoto *et al*., 2002; Wen *et al*., 2003; Miyamoto *et al*., 2004). However, neither upstream nor downstream intact IS*1470* sequences flank the plasmid *cpe* gene of type A isolates. Instead, about 70% of type A isolates carrying a *cpe* plasmid have defective IS*1470*-like sequences downstream of their *cpe* gene; the remaining 30% of type A isolates carrying a *cpe* plasmid have IS*1151* sequences downstream of their *cpe* gene (Miyamoto *et al*., 2002; Wen *et al*., 2003; Miyamoto *et al*., 2004). Recent studies (Fisher *et al*., 2005) found that the ~75 kb plasmid with the *cpe*-IS*1151* locus typically also carries a *cpb2* gene encoding beta2 toxin. In contrast, the ~75 kb plasmid with the *cpe*-IS*1470*-like locus lacks a *cpb2* gene; diversity clearly exists among the *cpe* plasmids of type A isolates.

As already mentioned, type E isolates usually carry a plasmid with silent *cpe* sequences located near their functional iota toxin genes (Billington *et al*., 1998). IS*1151* sequences are also present in this region of the type E virulence plasmid (Billington *et al*., 1998). Together, those observations suggest the typical type E virulence plasmid may have resulted from insertion of a genetic element containing iota toxin–like genes, which can be found in several pathogenic clostridial species, near the promoter of a plasmid *cpe* gene in a type A isolate. This insertion event inactivated that plasmid *cpe* gene, permitting the accumulation of additional mutations in the ribosome-binding site and ORF of the (now silent) *cpe* sequences in this newly created type E isolate.

The *cpe* mutations present in most/all type E isolates are very similar (Billington *et al*., 1998), suggesting that the progenitor type E virulence plasmid has spread to other *C. perfringens* type A isolates. That observation provided an early suggestion that plasmids carrying *cpe* sequences might be transferable between *C. perfringens* isolates, which has been directly confirmed in a recent study (Brynestad *et al*., 2001) demonstrating that the *cpe* plasmid from type A isolate F4969 can conjugatively transfer among type A isolates.

### *Relationships between* cpe *genotypes and GI disease*

It is now well established that type A isolates causing *C. perfringens* type A food poisoning typically carry a chromosomal *cpe* gene, while type A isolates causing CPE-associated AAD/SD usually have a plasmid-borne *cpe* gene (Cornillot *et al*., 1995; Collie and McClane, 1998; Sparks *et al*., 2001; Miyamoto *et al*., 2002; Wen *et al*., 2003; Miyamoto *et al*., 2004). Possible explanations are now emerging for these strong associations between *cpe* genotypes (i.e., whether an isolate carries a plasmid-borne or chromosomal *cpe* gene) and specific CPE-associated human GI diseases.

Two observations offer at least partial explanations for the common involvement of chromosomal *cpe* isolates in *C. perfringens* type A food poisoning. First, type A isolates carrying a chromosomal *cpe* gene are much more heat-resistant than type A isolates carrying a plasmid *cpe* gene (Sarker *et al*., 2000), a trait that likely favors their survival in the incompletely cooked or inadequately held foods that cause food poisoning outbreaks. Second, recent studies (Wen and McClane, 2004) have established that meat and poultry, which are the most common food vehicles for *C. perfringens* type A food poisoning, are much more likely to be contaminated with type A isolates carrying a chromosomal *cpe* gene rather than a plasmid *cpe* gene.

The strong linkage between type A isolates carrying a *cpe* plasmid and CPE-associated AAD/SD also appears to be multifactorial. For example, while *C. perfringens* type A food poisoning results from ingestion of greater than $10^6$–$10^7$ *C. perfringens* vegetative cells/ml, some evidence suggests that CPE-associated AAD/SD often results from ingestion of a low *C. perfringens* dose (Carman, 1997). Under low infecting doses, establishment of CPE-associated AAD/SD might be assisted by the presence of the *cpe* gene on a conjugative plasmid (as already shown for SD isolate F4969 (Brynestad *et al*., 2001)), which would allow ingested *cpe*-positive *C. perfringens* cells to transfer their *cpe* plasmid to the *cpe*-negative *C. perfringens* commonly found in the normal human GI flora. This putative *in vivo cpe* plasmid transfer would convert the recipient normal flora *C. perfringens* isolates to enteric virulence, thus amplifying the original infective dose and thereby increasing the probability of colonization and disease.

Putative *in vivo* transfer of the *cpe* plasmid potentially offers a second contribution to AAD/SD, i.e., this process will result in the presence of the *cpe* gene in normal flora *C. perfringens* isolates. As normal flora *C. perfringens* are presumably under selective pressure for persistence in the human GI tract, conjugative acquisition of the *cpe* plasmid by colonization-proficient normal GI flora *C. perfringens* might help explain clinical observations (Carman, 1997), indicating that the GI symptoms of CPE-associated AAD/SD typically persist longer than the GI symptoms of *C. perfringens* type A food poisoning.

Finally, it has already been mentioned that recent studies (Fisher *et al*., 2005) found greater than 75% of all type A isolates carrying a plasmid-borne *cpe* gene also express beta2 toxin while fewer than 15% of chromosomal *cpe* isolates express beta2 toxin (Fisher *et al*., 2005). Since purified beta2 toxin can kill the human enterocyte-like CaCo-2 cell line (Fisher *et al*., 2005), the ability

of most type A isolates carrying a plasmid *cpe* gene to produce two potentially enterically active toxins, i.e., CPE and beta2, could help explain why the GI symptoms of CPE-associated AAD/SD are typically more severe than those of *C. perfringens* type A food poisoning.

## CPE expression

### *Features of CPE expression*

Since the pioneering work of Duncan's group back in the 1970s (Melville *et al*., 1997), a strong linkage between CPE expression and sporulation has been apparent. Duncan's early studies showed that a stage 0 sporulation mutant of *C. perfringens* food poisoning isolate NCTC8798 lost the ability to produce CPE. Western blot studies (Czeczulin *et al*., 1993) further confirmed a relationship between sporulation and CPE production by demonstrating more than 1500-fold higher CPE production by sporulating vs. vegetative cells of food poisoning isolate NCTC 8239. Other Western blot studies (Collie *et al*., 1998) later demonstrated that CPE expression from both the chromosomal and plasmid-borne *cpe* genes is strongly sporulation-associated. Two recent studies (Huang *et al*., 2004; Varga *et al*., 2004) using genetic approaches added further support to a relationship between CPE expression and sporulation by showing that isogenic SpoOA and CcpA mutants prepared in the SM101 CPE-positive food poisoning derivative are completely or partially (respectively) blocked for both sporulation and CPE production.

Another pathogenesis-relevant feature of CPE expression is the extremely large amount of this toxin produced by many type A isolates. CPE often accounts for greater than 15% of the total protein present inside sporulating cells of those *C. perfringens* isolates (Czeczulin *et al*., 1993; Collie *et al*., 1998).

### *Regulation of CPE expression*

The sporulation-associated nature of CPE expression involves strong regulation at the transcriptional level. Northern blot and RNA slot blot studies (Melville *et al*., 1994; Czeczulin *et al*., 1996) detected significant levels of *cpe* mRNA in sporulating, but not vegetative cultures, of *C. perfringens* food poisoning isolates. Those Northern blot analyses (Czeczulin *et al*., 1996) also revealed that *cpe* mRNA is ~1.2 kb in size, which is consistent with transcription of the *cpe* gene as a monocistronic message. Monocistronic transcription of the *cpe* gene is compatible with the (previously mentioned) identification of (i) several promoters located ~200 bp upstream of the *cpe* initiation codon and (ii) a putative transcriptional terminator loop/oligo dT tract that is present 36 bp downstream of the *cpe* termination codon (Czeczulin *et al*., 1993; Zhao and Melville, 1998).

The strong association between sporulation and CPE expression likely involves DNA sequences present immediately upstream of each of the three *cpe* promoters (Zhao and Melville, 1998). Those upstream sequences share homology with *Bacillus cereus* sequences associated with SigE-dependent or SigK-dependent promoters, which is significant since SigE and SigK are sporulation-associated alternative sigma factors that become active in mother cells during the sporulation of *B. cereus* (Zhao and Melville, 1998). Presumably, these two alternative sigma factors function similarly during the sporulation of *C. perfringens* to activate transcription of some sporulation-associated genes (including *cpe?*).

The high levels of CPE produced by many enterotoxigenic *C. perfringens* isolates (see previous section) appear attributable, at least in part, to the multiple promoters located upstream of the *cpe* gene. This hypothesis receives direct support from recent studies (Chen *et al*., 2004), demonstrating that *cpe* promoters can also drive strong, sporulation-associated production of foreign proteins (e.g., HIV proteins) in recombinant *C. perfringens* (Note: *cpe* promoter-driven foreign antigen expression represents a key component of recent studies (Chen *et al*., 2004) aimed at developing attenuated *C. perfringens* constructs as possible vaccine delivery vectors).

An older study (Labbe and Duncan, 1977) determined that *cpe* mRNA may have an unusually long (~45 min) half-life. That observation raises the possibility of posttranscriptional effects, particularly message stability, also contributing to the strong production of CPE that often occurs during sporulation.

### *Release of CPE from sporulating* **C. perfringens** *cells*

The *cpe* gene does not encode a signal peptide for CPE secretion (Czeczulin *et al*., 1993). Instead, CPE accumulates in the cytoplasm of the mother cell during sporulation and is finally released into the GI tract only at the completion of sporulation, i.e., when the mother cell lyses to release its now mature endospore.

## THE INTESTINAL ACTION OF CPE

CPE is considered an enterotoxin because it induces fluid and electrolyte losses from the GI tract of many mammalian species (McDonel, 1986). Animal studies indicate that the small intestine is the principal target organ for CPE (McDonel, 1986). CPE affects all small intestinal regions, but is most active on the ileum

(McDonel, 1986). The rabbit colon does not significantly respond to CPE, even though isolated rabbit colonic cells bind the toxin at high levels (McDonel and Demers, 1982). Whether CPE affects the human colon is not yet clear.

CPE acts very quickly on the small intestines, with high toxin doses causing visible ileal damage within 30 min of treatment (Sherman *et al*., 1994). Two observations indicate that this initial histopathologic damage, which starts at the villus tips, is responsible for starting CPE-induced intestinal fluid/electrolyte transport alterations: (i) the onset of fluid/electrolyte transport alterations occurs concurrently with the development of damage in the CPE-treated rabbit ileum (Sherman *et al*., 1994), and (ii) only those CPE doses capable of inducing intestinal damage can induce fluid/electrolyte transport alterations (McDonel and Duncan, 1975).

CPE exerts "biphasic" alterations in ileal fluid/electrolyte transport (Sherman *et al*., 1994). Initially, the CPE-treated ileum shows only an inhibition of fluid/electrolyte absorption; as described previously, this initial absorption inhibition is probably attributable to the start of histopathologic damage in the CPE-treated ileum. However, as treatment time increases, frank ileal fluid/electrolyte secretion develops in the CPE-treated ileum. This intestinal secretion probably reflects a combination of (i) secondary effects of the enterotoxin on intestinal paracellular permeability (see next section) and (ii) continued development of histopathologic damage that eventually desquamates the epithelium of the CPE-treated ileum and causes severe villus shortening (Sarker *et al*., 1999).

## THE CELLULAR ACTION OF CPE

### Step one: CPE binds to cells, forming a small, SDS-sensitive complex

Recent studies (Hardy *et al*., 2001) demonstrated that the addition of CPE to protein-free artificial membranes can produce cation-selective pores. That observation clearly indicates protein receptors are not absolutely required for CPE action. However, those artificial membrane studies used very high CPE concentrations to obtain pores that developed much slower than occurs in naturally CPE sensitive cells (see CPE-Induced Plasma Membrane Permeability Alterations).

Considerable evidence indicates that protein receptors do play an important role in initiating CPE action under pathophysiologic conditions. For example, cultured mouse fibroblasts, which are unable to specifically bind CPE due to the lack of receptors, fail to respond to the moderate CPE concentrations typically present in the intestines during GI disease (Katahira *et al*., 1997a; Katahira *et al*., 1997b; Singh *et al*., 2000). However, those cells specifically bind CPE and become highly sensitive to this toxin if transfected to express certain claudins (Katahira *et al*., 1997a; Katahira *et al*., 1997b).

Besides demonstrating the importance of protein receptors for normal CPE action, the transfectant results of Katahira *et al*. also indicate that some claudins can serve as functional CPE receptors. Claudins are a recently discovered family (more than 20 members) of small (about 22 kDa) proteins that play key roles in mediating both the barrier and gate functions of epithelial tight junctions (TJs). Interestingly, only certain claudins can serve as functional CPE receptors. Mouse fibroblast transfectant studies (Fujita *et al*., 2000) indicate that claudins -3, -4, -6, -7, -8, and -14 can bind CPE, while claudins -1, -2, -5, and -10 cannot bind CPE. The importance of claudins as pathophysiologic CPE receptors for enterocytes has not yet been addressed.

Immediately upon binding to claudin (and/or other) protein receptors, CPE localizes in a small (about 90 kDa) SDS-sensitive complex (Wieckowski *et al*., 1994). Formation of the small CPE complex (Figure 45.3) appears important for the enteuotoxin's action since this complex is formed by every CPE-sensitive cell line tested to date. The complete composition and stoichiometry of small CPE complex are not yet clear, but immunoprecipitation analyses (Wieckowski *et al*., 1994) demonstrated this complex contains both CPE and a mammalian protein that runs at about 45–50 kDa on SDS-PAGE. Whether the ~45–50 protein represents aggregated claudins, an alternative CPE receptor, or a co-receptor that (along with claudins) interacts with CPE, has not yet been determined.

When present in the small complex, CPE apparently becomes trapped on the surface of the host cell membrane (Wieckowski *et al*., 1998). For example, CPE localized in small complex shows relatively limited dissociation from mammalian cells. However, CPE in small complex does not appear to be internalized or to insert in lipid bilayers as this toxin remains highly susceptible to external protease treatments.

### Step two: CPE becomes localized in large, SDS-resistant complexes

At 4°C, CPE binds and forms small complexes, but does not induce any cytotoxic effects (McClane and Wnek, 1990). However, if cells containing CPE bound at 4°C are subsequently warmed to 37°C, cytotoxicity

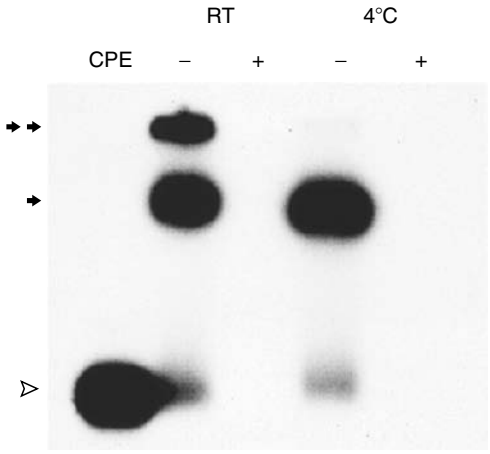



**FIGURE 45.3** Formation of CPE-containing complexes at room temperature (RT) or 4°C. For this experiment, $^{125}$I-CPE was incubated with brush border membranes in the presence (+) or absence (–) of excess native toxin. After extraction with Triton X-100, lysates were subjected to non-denaturing electrophoresis and gels were then autoradiographed. The white arrow, outlined in black, represents free $^{125}$I-CPE dissolved in Triton X-100 that had never been exposed to membranes. The single, solid black arrow depicts the location of the ~ 90 kDa small complex, while the double arrow indicates migration of higher $M_r$ complexes. Reproduced with permission from (Wieckowski *et al*., 1994).

quickly develops. Concurrent with that development of cytotoxicity, much of the CPE bound to those cells shifts (Figure 45.3) from the small complex to higher $M_r$ material (McClane and Wnek, 1990; Wieckowski *et al*., 1994). Collectively, these observations indicate (i) although important, formation of the small CPE complex is insufficient to induce cytotoxicity, (ii) small complex is a precursor for formation of the higher $M_r$, CPE-containing material, and (iii) some or all of the higher $M_r$, CPE-containing material is essential for cytotoxicity.

The importance of higher $M_r$, CPE-containing material for CPE-induced cytotoxicity receives further support from deletion mutagenesis, random mutagenesis, and site-directed mutagenesis studies (Kokai-Kun and McClane, 1997; Kokai-Kun *et al*., 1999; Smedley and McClane, 2004). Collectively, those studies identified several CPE fragments or point mutants that can bind CPE and form small complexes, yet fail to form the higher $M_r$, CPE-containing material; in all cases, those CPE fragments/point mutants are non-cytotoxic. In addition, some CPE fragments were identified that bind normally, yet form increased amounts of the higher $M_r$, CPE-containing material; those fragments also consistently exhibit two-to three-fold greater cytotoxic activity than native CPE.

Recent Western blot studies (Singh *et al*., 2000) examined suspensions of human enterocyte-like CaCo-2 cells treated with CPE at 37°C. Those studies successfully resolved (Figure 45.4) the higher $M_r$, CPE-containing material into two major SDS-resistant CPE species of about 155 kDa and about 200 kDa. A minor CPE-containing, SDS-resistant species of about 135 kDa was also sometimes detected during these studies. An important breakthrough in understanding the composition of these large CPE complexes occurred when it was found (Singh *et al*., 2000) that the TJ protein occludin is present in the ~ 200 kDa complex, but is not a component of the ~155 kDa complex (Figure 45.4). Furthermore, CPE treatment was shown (Singh *et al*., 2001) to slowly induce internalization of occludin from TJs into the cytoplasm, an effect that could contribute to CPE-induced disease (see "Consequences of CPE-Induced Cell Death: TJ Effects").

In CaCo-2 cell monolayers grown in Transwell cultures (to produce a polarized epithelium) and then treated with CPE on their apical surface, formation of the ~200 kDa complex requires about two hours (Singh *et al*., 2001). In contrast, 20 min of CPE treatment on the apical surface is enough time for these CaCo-2 cell Transwell cultures to form the ~ 155 kDa complex and exhibit the substantial $^{86}$Rb-release indicative of CPE-induced cytotoxicity (see next section). Thus, it appears that, under experimental conditions resembling *in vivo* GI disease, formation of the ~155 kDa complex is

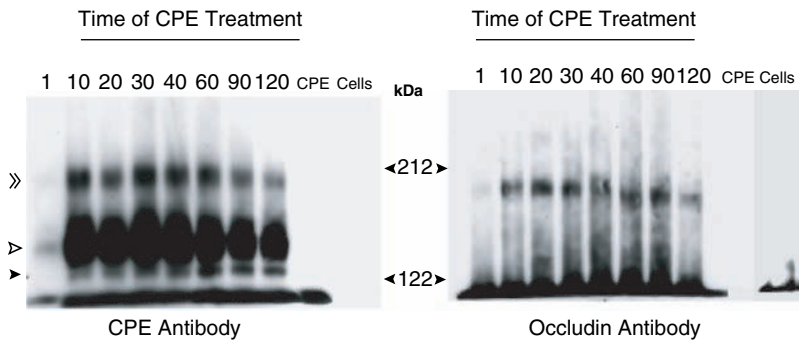



**FIGURE 45.4** The kinetics of CPE large complex formation in suspensions of CaCo-2 cells. CPE was added to a suspension of CaCo-2 cells for the indicated time at 37°C. After washing to remove unbound toxin, cells were lysed with SDS. The resultant lysates were analyzed by SDS-PAGE (no sample boiling) using 4% acrylamide gels. The gels were then Western blotted with either CPE antibodies (left) or occludin antibodies (right). The double, open, and closed arrows represent the migration of the ~ 200 kDa, ~155 kDa, or ~135 kDa complexes, respectively. Reproduced with permission from (Singh *et al*., 2000).

sufficient to initiate CPE-induced cytotoxicity in polarized monolayers of human enterocyte-like CaCo-2 cells.

Additional studies are now underway to establish the protein composition and stoichiometry of each CPE complex.

## Step three: CPE causes plasma membrane permeability changes

It was first shown back in 1979 that CPE disrupts normal plasma membrane permeability in sensitive mammalian cells (McDonel and McClane, 1979). Within 15 min of CPE treatment, sensitive mammalian cells become dramatically more permeable to small molecules (less than 200 Daltons), including ions and amino acids (McClane and McDonel, 1980, 1981; McClane, 1984; Horiguchi *et al*., 1985). Over 1–2 hours of CPE treatment, cells gradually become permeable to larger molecules up to about 3000 Daltons (McClane and McDonel, 1980; McClane, 1984).

Substantial evidence now indicates that CPE-induced permeability alterations are caused by pore formation. Electrophysiology studies demonstrated that CPE forms cation-selective pores in both artificial membranes and CaCo-2 cells (Hardy *et al*., 1999; Hardy *et al*., 2001). Furthermore, plasma membranes offer CPE in large complexes substantial protection from external proteases (Wieckowski *et al*., 1998), which is fully consistent with substantial portions of the CPE protein inserting into lipid bilayers to form pores after the toxin becomes localized in the large CPE-containing complexes responsible for CPE-induced cytotoxicity.

## Step four: CPE-induced cell death

### *Brief introduction to mammalian cell death pathways*

Mammalian cells sometimes respond to lethal insults by triggering apoptosis, a programmed cell death involving activation of specific proteases (often caspase 3/7), nuclear condensation, and cleavage of DNA into a ladder-like cleavage pattern of 200 bp increments (Majno and Joris, 1995). Apoptosis often (though not always) involves mitochondrial membrane depolarization and subsequent cytochrome C release. When it occurs, mitochondrial membrane depolarization can either result from caspase activation (i.e., be a caspase-dependent process) or can itself trigger caspase activation (i.e., be a caspase-independent process).

Alternatively, mammalian cells exposed to extreme insults can develop oncosis (formerly referred to as necrosis), which is a chaotic breakdown of cellular integrity accompanied by random cleavage of DNA in a "sheared" pattern (Majno and Joris, 1995). Oncosis does not involve either mitochondrial membrane depolarization or cytochrome C release and often can be transiently blocked by glycine. A special form of oncosis, named pyrotosis, requires caspase-1 activation (Cookson and Brennan, 2001).

### *Characterization of CPE-induced cell death*

Nearly 25 years ago, it was determined that CPE is highly cytotoxic for most mammalian epithelial cells (Matsuda and Sugimoto, 1979; McClane and McDonel, 1979). When treated with this toxin, cells rapidly lose viability and experience a simultaneous shutdown of DNA, RNA, and protein synthesis. Coincident with those effects, the CPE-treated cells develop substantial morphologic damage (cell rounding, bleb formation, and detachment).

Recent studies (Chakrabarti *et al*., 2003; Chakrabarti and McClane, 2005) investigated the cell death pathways that are activated by CPE treatment of CaCo-2 cell monolayers. As summarized in Table 45.2, a 1 μg/ml CPE dose causes CaCo-2 cells to develop morphologic damage, DNA fragmentation in a ladder-like cleavage pattern, and caspase 3/7 activation within 60 min Those effects could all be blocked by caspase-3/7 inhibitors, but not by caspase-1 or oncosis inhibitors. The 1 μg/ml CPE dose also induces rapid mitochondrial membrane depolarization and cytochrome C release in CaCo-2 cells, but those mitochondrial-related effects cannot be inhibited by any caspase inhibitors or glycine.

In contrast, CaCo-2 cells treated with a 10 μg/ml CPE dose for 30 min develop morphologic damage and a smeared pattern of DNA cleavage that can be transiently blocked by glycine, but not by any caspase inhibitors (even though modest caspase 1 activation occurs in these cells). This high CPE dose has no effect on mitochondrial membrane depolarization or cytochrome C release.

Collectively, these results indicate that a low (1 μg/ml) CPE dose induces mitochondrial membrane depolarization and cytochrome C release that causes a classic caspase 3/7-dependent apoptosis in CaCo-2 cells. In contrast, a high (10 μg/ml) CPE dose apparently induces oncosis in CaCo-2 cells. Although CPE-induced oncosis causes a modest activation of caspase 1, that effect is not required to obtain cell death, i.e., high CPE does not induce pyrotosis (the caspase 1-dependent form of oncosis). Finally, since CPE concentrations present in feces from victims of CPE-associated GI disease range from several ng/ml to greater than 100 μg/ml (Batholomew *et al*., 1985; Birkhead *et al*., 1988), both apoptosis and oncosis can probably contribute to the histopathologic damage that initiates CPE action *in vivo*.

**TABLE 45.2** Characteristics of CaCo-2 cell death induced by low (1 μg/ml) and high (10 μg/ml) CPE doses

| Characteristic | Caco-2 cells treated with low CPE dose | CaCo-2 cells treated with high CPE dose |
|---|---|---|
| Cell rounding/detachment (morphologic damage) | Develop within 60 min[1] | Develop within 30 min[2] |
| DNA cleavage | Ladder-like cleavage (develops within 60 min)[1] | Smeared cleavage (develops within 30 min)[2] |
| Caspase 3 activation | Strong (develops within 60 min)[1] | None |
| Caspase 1 activation | None | Mild (within 30 min) |
| Mitochondrial membrane depolarization | Within 15 min | None |
| Cytochrome C release | Within 15–30 min | None |
| Calmodulin involvement | Yes | Yes |
| Calpain involvement | Yes | Yes |
| Calpain activation | Mild | Strong |
| Calcium influx | Slow and mild | Fast and strong |
| Cell calcium levels | Slow, moderate increase | Fast, strong increase |

[1]Effect delayed by caspase 3/7 inhibitors
[2]Effect delayed by the oncosis inhibitor glycine
Results compiled from (Chakrabarti *et al.*, 2003; Chakrabarti and McClane, 2005).

### *The role of calcium in CPE-induced cell death*

Matsuda and Sugimoto (1979) first noted that extracellular $Ca^{2+}$ is important for obtaining CPE-induced morphologic damage, but that $Ca^{2+}$ requirement had never been explained prior to a recent study (Chakrabarti and McClane, 2005). That recent study first demonstrated that CaCo-2 cells treated with CPE in the absence of extracellular $Ca^{2+}$ still develop membrane permeability alterations, i.e., extracellular $Ca^{2+}$ requirement for CPE-induced cell death occurs after the enterotoxin has bound, formed the ~155 kDa large complex, and induced plasma membrane permeability alterations. Furthermore, evidence was presented demonstrating that extracellular $Ca^{2+}$ is specifically necessary for the normal development of all events linked to either CPE-induced apoptosis or oncosis (see preceding section).

Chakrabarti and McClane also showed that the extracellular $Ca^{2+}$ requirement for CPE-induced cell death involves influx of $Ca^{2+}$, a potent second messenger capable of mediating cell death. As indicated in Table 45.2, CPE-induced $Ca^{2+}$ influx quickly raises cell calcium levels in CaCo-2 cells. Both the onset and magnitude of those cellular calcium increases are CPE dose-dependent, i.e., a faster and stronger increase in cell calcium levels occurs using a 10 vs. 1 μg/ml CPE dose.

Increases in cellular $Ca^{2+}$ levels appear to play a pivotal role in normal CPE-induced cell death. For example, caspase inhibitors and glycine (which are capable of blocking CPE-induced apoptosis or oncosis, respectively) fail to block CPE-induced increases in CaCo-2 cell $Ca^{2+}$ levels (Chakrabarti and McClane, 2005), arguing that increased calcium levels are not merely a consequence of CPE-induced cell death. Furthermore, Chakrabarti and McClane (2005) showed that the cell death pathway activated by a given CPE dose can be experimentally shifted from apoptosis to oncosis, (or vice versa) simply by manipulating the calcium content of treatment buffers to obtain greater (or lesser) increases in cell calcium levels, i.e., cell calcium levels directly select which death pathway becomes activated in a CPE-treated cell.

How do CPE-induced changes in cellular $Ca^{2+}$ levels trigger cell death? As shown in Table 45.2, inhibitor studies (Chakrabarti and McClane, 2005) indicate that two calcium-binding cytosolic proteins, i.e., calmodulin and calpain, play essential roles in CPE-induced apoptosis and oncosis. Furthermore, stronger and faster calpain activation occurs in CaCo-2 cells treated with 10 vs. 1 μg/ml CPE doses. The pathway by which calcium-induced activation of calpain and calmodulin leads to death of the CPE-treated cell is currently under study.

### *Consequences of CPE-induced cell death: Initiation of GI disease*

CPE-induced apoptosis and oncosis produce morphologic damage, which probably initiates the histopathologic damage responsible for starting fluid and electrolyte transport alterations in the CPE-treated intestines.

### *Consequences of CPE-induced cell death: Tight junction effects*

The morphologic damage resulting from CPE-induced apoptosis and oncosis should also expose the basolateral

membrane of both intoxicated and adjacent (but still healthy) enterocytes. Exposing enterocyte basolateral membranes probably has pathophysiologic relevance since it would provide free (unbound) CPE access to (previously hidden) CPE receptors present on basolateral membranes. This effect could be important since Transwell CaCo-2 cell studies detected considerably more CPE receptors on the basolateral vs. apical surface of these enterocyte-like, human polarized cells (Singh *et al*., 2001).

CPE binding to receptors on the basolateral surface of enterocyte-like cells (and, probably, enterocytes) is likely to have two effects (Singh *et al*., 2001). First, it should increase ~155 kDa complex formation, further promoting cell death. Second, it should allow bound CPE to interact with (previously inaccessible) occludin, another TJ protein. Studies with CaCo-2 cells (Singh *et al*., 2001) indicate that, when CPE binds to basolateral receptors, the ~200 kDa, occludin-containing complex forms within 1 h (this complex also forms, although considerably more slowly, when CPE binds to apical receptors).

Formation of the ~200 kDa CPE complex coincides with considerable removal of occludin from the TJ into the cytoplasm (Singh *et al*., 2001). Other studies (Sonoda *et al*., 1999) have shown that a binding-capable CPE fragment (and presumably native CPE) can induce internalization of some claudins. Collectively, these studies suggest that removal of claudins and occludin from TJs probably helps explain CPE's ability to damage TJs (McClane, 2000), which (in turn) likely explains the increased paracellular permeability observed (Sonoda *et al*., 1999) in polarized monolayers treated with CPE fragments (and, presumably, native CPE). Those CPE-induced increases in paracellular permeability likely contribute to the fluid/electrolyte secretion that occurs later in CPE action.

### *Consequences of CPE-induced cell death: Inflammation?*

Inflammation contributes to the symptoms of many infectious GI diseases, e.g., *C. difficile*-associated infections. Since oncosis is proinflammatory, the discovery that high CPE doses kill cells via oncosis suggests that intestinal inflammation could also contribute to the diarrheal and cramping symptoms of CPE-associated GI disease. As mentioned earlier, this possibility is supported by studies determining that fecal CPE concentrations from *C. perfringens* food poisoning victims often exceed the 10 μg/ml CPE dose found to induce oncosis in CaCo-2 cells. Studies are currently underway to directly evaluate the potential proinflammatory properties of CPE.

## SUMMARY: A CURRENT MODEL FOR CPE ACTION

The results described above indicate that CPE's intestinal effects are the end result of a multi-step action. The current model for CPE action proposes this process starts (Figure 45.5A) with the binding of CPE to claudin receptors (perhaps together with a co-receptor?) to form the 90 kDa small complex. Through a still unidentified process, the small CPE complex then interacts with other proteins to form the ~155 kDa CPE complex. That complex, or perhaps oligomers of that complex, forms pores that render the plasma membrane permeable to small molecules, including $Ca^{2+}$. Low CPE doses cause a moderate $Ca^{2+}$ influx that, via modest activation of calmodulin and calpain, induces mitochondrial membranes to release cytochrome C, which leads to caspase 3/7 activation and cell death from classical apoptosis. At higher CPE doses, a massive $Ca^{2+}$ influx occurs that causes, via strong activation of calmodulin and calpain, cell death via oncosis.

As indicated in Table 45.B, both oncosis and apoptosis result in morphologic damage that exposes the basolateral membranes of the intoxicated cell and adjacent neighbors. This allows CPE to bind to newly exposed basolateral claudin receptors; the basolaterally bound toxin then forms more ~155 kDa complex and also interacts with occludin to form the ~200 kDa complex. Formation of the ~200 kDa complex triggers internalization of occludin (and possibly CPE and claudins) to damage TJs. As shown in Figure 45.6, the CPE-damaged TJs can no longer properly regulate paracellular permeability; along with gross desquamation of the intestinal epithelium and severe villus shortening, altered paraceullar permeability likely contributes to CPE-induced diarrhea. When high CPE doses cause enterocytes to die from oncosis, inflammation may also contribute to diarrhea and other GI symptoms (not shown).

Finally, new evidence (Fisher *et al*., 2005) indicates that both CPE and beta2 toxin may be present in the intestines during most cases of CPE-associated non-foodborne human GI disease. Those two enterically active toxins could act together to cause the more severe and long-lasting symptoms typical of cases of CPE-associated non-foodborne GI diseases vs. cases of *C. perfringens* type A food poisoning.

## CPE STRUCTURE/FUNCTION RELATIONSHIPS

Because CPE lacks significant sequence homology with other bacterial toxins and the 3D structure of this toxin

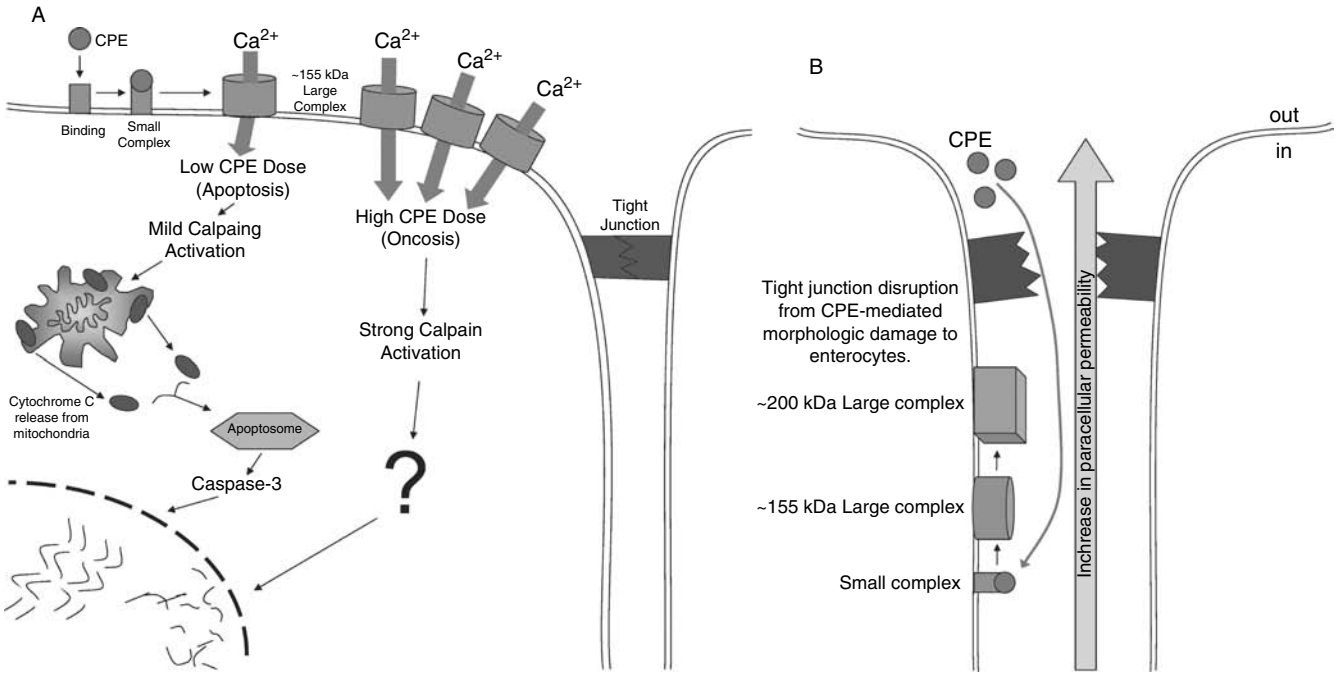



**FIGURE 45.5** Model for CPE action: early steps. A. Steps leading to CPE-induced cell death: CPE binds to receptors (possibly claudins) to form small complex. The small complex then interacts with additional proteins to form an about 155 kDa large complex whose formation leads to calcium influx, probably through a CPE-containing pore. At low CPE doses, modest calcium influx leads to activation of a classical caspase-3 dependent apopotosis mediated by mitochondria. High CPE doses cause a massive calcium influx that kills cells via oncosis. Both death pathways are mediated by activation of calpain and calmodulin (not shown in figure) and lead to morphologic damage of the CPE-treated cell. B. Secondary consequences of CPE-induced morphologic damage. Morphologic damage induced by CPE-induced cytotoxicity exposes the basolateral surface on the intoxicated cell and adjacent cells. The basolateral surface contains many CPE receptors; exposure of this surface allows more CPE binding to form more ~155 kDa complex formation and also permits initial formation of the about 200 kDa complex containing occludin. Formation of the ~200 kDa complex probably facilitates further tight junction damage.

has not yet been solved, studies of CPE structure/function relationship studies have relied upon mutagenesis approaches. Results from those structure/function studies (summarized in Figure 45.7) indicate that, like many bacterial toxins, binding and toxicity activities segregate to different regions of the CPE protein.

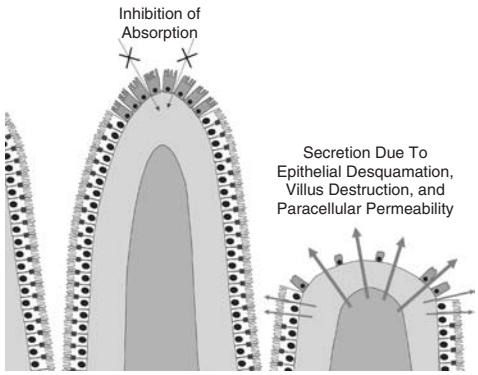



**FIGURE 45.6** Summary of the intestinal effects of CPE. Left, CPE initially kills cells at the villus tip (darkened cells in the figure), inhibiting absorption of fluids and electrolytes (Xs). Right, secretion of fluids and electrolytes develops later due to (big arrows) severe desquamation of the intestinal epithelium/villus shortening and/or (small arrows) increased CPE-induced paracellular permeability changes due to tight junction damage.

## The CPE receptor binding region

Early studies, where CPE was chemically cleaved at its single cysteine (residue 186), suggested that the

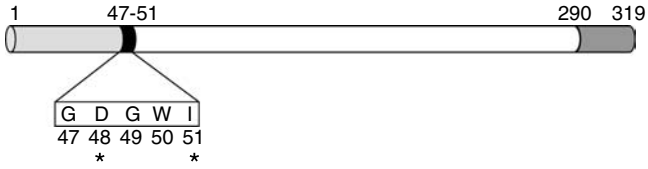



**FIGURE 45.7** CPE structure/function relationships. The N-terminal 44 amino acids of CPE are not important for cytotoxicity; their removal (which may be induced by intestinal proteases during GI disease) increases CPE potency by promoting large complex formation. The region between amino acids 47 to 51 of the native enterotoxin is important for formation of the ~155 kDa and ~200 kDa large CPE complexes, with the two residues (D48A and I51) marked by an asterisk that are specifically involved in this process. A receptor-binding region is located in the extreme C-terminus of CPE. This figure is compiled from numerous studies described in the structure/function section of the text.

C-terminal half of CPE might contain receptor-binding activity (Horiguchi *et al*., 1987). That assignment was rigorously confirmed by recombinant DNA approaches, which demonstrated that a recombinant CPE (rCPE) fragment consisting of enterotoxin amino acid residues 171 to 319 is an extremely efficient receptor-binding competitor against native CPE (Hanna *et al*., 1989). Subcloning approaches and synthetic peptide studies further localized this CPE receptor-binding activity to the C-terminal 30 amino acids of the native enterotoxin (Hanna *et al*., 1991).

### The CPE cytotoxicity region

The early structure/function studies cited above also found that, despite their ability to bind to receptors, C-terminal CPE fragments fail to induce any cytotoxicity (Hanna *et al*., 1989; Hanna *et al*., 1991). Those observations indicated that some sequences present in the N-terminal half of the enterotoxin are required for biologic activity.

However, those N-terminal cytotoxicity sequences do not include the extreme N-terminal amino acids of the native CPE protein. Early studies (Granum *et al*., 1981; Granum and Richardson, 1991) reported that removing the N-terminal 25 or 36 amino acids from CPE using the intestinal proteases trypsin or chymotrypsin, respectively, produces a two to three-fold more active toxin. This proteolytic activation, which may occur in the GI tract during disease, is not due to an enhancement of receptor binding (Hanna *et al*., 1992), which is consistent with localization of receptor-binding activity to the CPE C-terminus (see above).

Deletion mutagenesis studies (Kokai-Kun and McClane, 1997) later determined that CPE fragments lacking up to the N-terminal 44 amino acids of the native enterotoxin still exhibit this "activated toxicity" phenotype and also determined that this activation effect is due to enhanced formation of the SDS-resistant large CPE complexes. However, that study also found that deleting the first 52 amino acids produces an rCPE fragment that can bind to receptors but is completely nontoxic. This observation strongly suggested that the CPE region between residues D45 and G53 is important for toxicity. Random mutagenesis studies (Kokai-Kun *et al*., 1999) supported this conclusion by identifying a G49D rCPE mutant that is binding-proficient but non-toxic. Furthermore, those random mutagenesis studies also determined that the G49D rCPE mutant is specifically blocked for formation of large SDS-resistant complexes (including the ~155 kDa complex responsible for cytotoxicity), implying that the D45-G53 region of native CPE participates in formation of those complexes.

Recently, alanine scanning mutagenesis approaches have fine-mapped the D45-G53 region of CPE to identify the amino acid residues involved in cytotoxicity (Smedley and McClane, 2004). Those studies identified two CPE residues, D48A and I51A, as being specifically important for formation of the ~155 kDa and ~200 kDa large CPE complexes. Follow-up saturation mutagenesis studies (Smedley and McClane, 2004) then determined that both side chain length and charge are required for the D48 CPE residue to participate in large complex formation and cytotoxicity. Similar saturation mutagenesis analyses also found that residue size and/or hydrophobicity are necessary for the I51 CPE residue to participate in large complex formation and cytotoxicity.

## DEVELOPMENT OF A CPE VACCINE?

Epitope mapping studies, where C-terminal rCPE fragments were reacted with CPE-specific monoclonal antibodies, localized the linear epitope recognized by MAb3C9 to the extreme C-terminus of the enterotoxin (Hanna *et al*., 1992). Since MAb3C9 is a neutralizing antibody that blocks the binding of the enterotoxin to its receptor (Wnek *et al*., 1985), this finding provides additional evidence for the invol vement of the C-terminal CPE region in receptor binding.

Determining that a neutralizing linear epitope is present in non-cytotoxic C-terminal CPE fragments also opened the possibility of using those toxin fragments as CPE vaccine candidates. The vaccine potential of C-terminal CPE fragments received support by conjugating a 30-mer synthetic peptide (corresponding to the extreme C-terminal CPE sequence) to a thryoglobulin carrier (Mietzner *et al*., 1992). Mice immunized i.p. with that conjugate developed high titers of CPE-neutralizing serum IgG antibodies, while mice immunized with thyroglobulin alone failed to develop any protective antibody response against CPE.

While these initial results are promising, additional work is required to fully evaluate the vaccine potential of C-terminal CPE fragments in protecting against CPE-mediated intestinal disease. For example, can these fragments induce the IgA response that is probably required to prevent CPE-mediated GI disease? Such studies will be pursued only when a clear need is demonstrated for a CPE medical or veterinary vaccine.

## POTENTIAL THERAPEUTIC USE OF CPE

More than a decade of clinical experience using botulinum toxins indicates that even the most potent clostridial toxins can be safely harnessed for therapeutic use. Recent studies suggest similar potential use of CPE as a therapeutic agent against certain tumors.

The impetus for this work stemmed from studies indicating that many pancreatic, breast, and prostate cancers overexpress claudin-3 and/or claudin-4 (Long *et al*., 2001; Michl *et al*., 2001; Kominsky *et al*., 2004). The fact that those two claudins are high-affinity CPE receptors (Katahira *et al*., 1997a; Katahira *et al*., 1997b; Sonoda *et al*., 1999) suggested that the enterotoxin might be useful as a new modality for treating some or all of those cancers.

Recent results from several lab studies now support that hypothesis (Long *et al*., 2001; Michl *et al*., 2001; Kominsky *et al*., 2004). For example, pancreatic, prostate, and breast cancer cells expressing high levels of claudin CPE receptors were found to be highly sensitive to the enterotoxin *in vitro*. In contrast, cancer cells failing to express claudins are CPE-insensitive. Perhaps even more exciting are results using animal tumor models (Michl *et al*., 2001; Kominsky *et al*., 2004). For example, intratumoral injections of CPE into xenografts of panc-1 cells (human pancreatic cancer cells, Figure 45.8) or T47D cells (human breast cancer cells, not shown) cause significant tumor shrinkage, accompanied by necrosis. Equally important, mice carrying the tumor xenografts treated with CPE showed no ill effects from their therapy (Michl *et al*., 2001).

While work continues towards transitioning these preliminary findings to the clinic, obvious potential challenges remain to be addressed. For example, does the widespread distribution of claudin CPE receptors on epithelial cells restrict this approach to treatment of solid tumors? Will the development of tumor resistance to CPE treatment become a major problem? Nonetheless, growing momentum supports the potential for CPE (or binding-capable CPE derivatives) to eventually find clinical application as an antitumor agent.

## CONCLUSION

This chapter has emphasized the relatively unique nature of CPE, from its sporulation-associated expression to its ability to interact with TJ proteins to produce pores. The coming years are likely to reveal important new insights into this intriguing protein, including CPE's three-dimensional structure, how this toxin interacts with tight junction proteins, and the composition and stoichiometry of CPE complexes. The potential application of CPE or *cpe* promoters for therapeutics and vaccines, respectively, appears promising; perhaps some day CPE will be a friend as well as a foe?

## ACKNOWLEDGMENTS

Preparation of this chapter was supported by Public Health Service grant AI 19844-22 from the National Institute of Allergy and Infectious Diseases and by

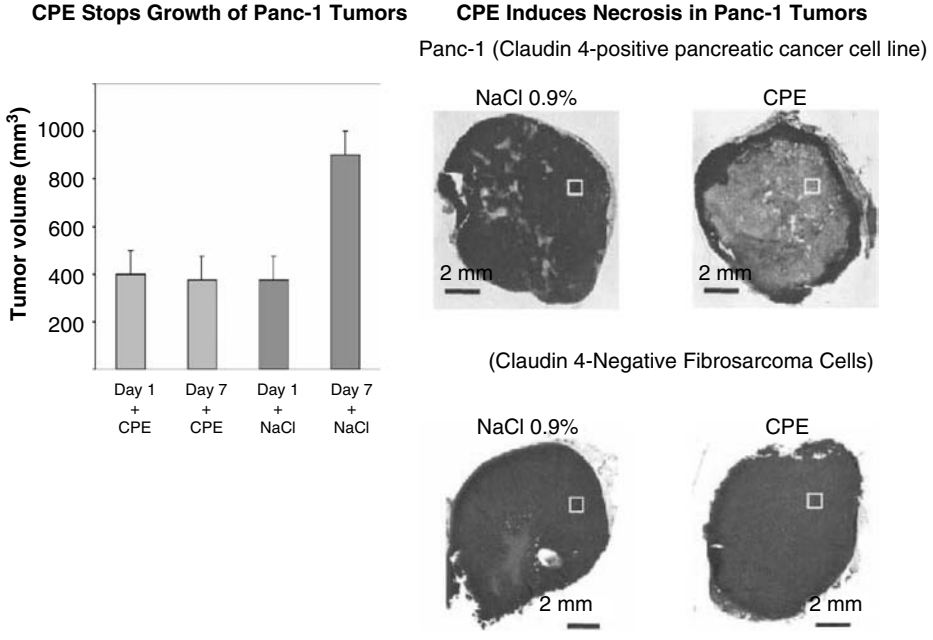



**FIGURE 45.8** CPE effects on pancreatic cancer cells expressing claudin-4. Left panel: Effect of one or seven day treatment of CPE or 0.9% NaCl on the volume of Panc-1 nude tumor xenograft models. Right panel: Histologic effects of treating claudin-4 expressing Panc-1 xenografts or claudin-4-negative HT-1080 xenografts with CPE or 0.9% NaCl. Necrotic areas appear as lighter shaded areas after H&E staining. Reproduced with permission from (Michl *et al*., 2001).

Ensuring Food Safety grant 2004-04066 from the U.S. Department of Agriculture. The author thanks James G. Smedley III, Sameera Sayeed, and Ganes Chakrabarti for preparing figures.

## REFERENCES


Batholomew, B.A., Stringer, M.F., Watson, G.N. and Gilbert, R.J. (1985). Development and application of an enzyme-linked immunosorbent assay for *Clostridium perfringens* type A enterotoxin. *J. Clin. Pathol*. **38,** 222–228.

Billington, S.J., Wieckowski, E.U., Sarker, M.R., Bueschel, D., Songer, J.G. and McClane, B.A. (1998). *Clostridium perfringens* type E animal enteritis isolates with highly conserved, silent enterotoxin sequences. *Infect. Immun*. **66,** 4531–4536.

Birkhead, G., Vogt, R.L., Heun, E.M., Snyder, J.T. and McClane, B.A. (1988). Characterization of an outbreak of *Clostridium perfringens* food poisoning by quantitative fecal culture and fecal enterotoxin measurement. *J. Clin. Microbiol*. **26,** 471–474.

Brynestad, S. and Granum, P.E. (1999). Evidence that Tn*5565*, which includes the enterotoxin gene in *Clostridium perfringens*, can have a circular form which may be a transposition intermediate. *FEMS Microbiol. Lett*. **170,** 281–286.

Brynestad, S., Sarker, M.R., McClane, B.A., Granum, P.E. and Rood, J.I. (2001). The enterotoxin (CPE) plasmid from *Clostridium perfringens* is conjugative. *Infect. Immun*. **69,** 3483–3487.

Brynestad, S., Synstad, B. and Granum, P.E. (1997). The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology* **143,** 2109–2115.

Carman, R.J. (1997). *Clostridium perfringens* in spontaneous and antibiotic-associated diarrhea of man and other animals. *Rev. Med. Microbiol*. **8,** *supplement 1*, S43–S45.

Chakrabarti, G. and McClane, B.A. (2005). The importance of calcium influx, calpain, and calmodulin for the activation of CaCo-2 cell death pathways by *Clostridium perfringens* enterotoxin. *Cell. Microbiol*. 7:129–146.

Chakrabarti, G., Zhou, X. and McClane, B.A. (2003). Death pathways activated in CaCo-2 cells by *Clostridium perfringens* enterotoxin. *Infect. Immun*. **71,** 4260–4270.

Chen, Y., Helmus, R., McClane, B.A., Hoffman, R., Watkins, S., Wehrli, T. and Gupta, P. (2004). Use of a *Clostridium perfringens* vector to express high levels of SIV p27 protein for the development of an oral SIV vaccine. *Virology*. 24:226–233

Collie, R.E., Kokai-Kun, J.F. and McClane, B.A. (1998). Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. *Anaerobe* **4,** 69–79.

Collie, R.E. and McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-foodborne human gastrointestinal diseases. *J. Clin. Microbiol*. **36,** 30–36.

Cookson, B.T. and Brennan, M.A. (2001). Pro-inflammatory programmed cell death. *Trends in Microbiol*. **9,** 113–114.

Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Carnard, B. and Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molec. Microbiol*. **15,** 639–647.

Czeczulin, J.R., Collie, R.E. and McClane, B.A. (1996). Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens. Infect. Immun*. **64,** 3301–3309.

Czeczulin, J.R., Hanna, P.C. and McClane, B.A. (1993). Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli. Infect. Immun*. **61,** 3429–3439.

Fisher, D.J., Miyamoto, K., Harrision, B., Akimoto, S., Sarker, M.R. and McClane, B.A. (2005). Association of Beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. *Molec. Microbiol*. 56:747–762.

Fujita, K., Katahira, J., Horiguchi, Y., Sonoda, N., Furuse, M. and Tskuita, S. (2000). *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction membrane protein. *FEBS Letters* **476,** 258–261.

Gibert, M., Jolivet-Reynaud, C. and Popoff, M.R. (1997). Beta2 toxin, a novel toxin produced by *Clostridium perfringens. Gene* **203,** 65–73.

Granum, P.E. and Richardson, M. (1991). Chymotrypsin treatment increases the activity of *Clostridium perfringens* enterotoxin. *Toxicon*. **29,** 445–453.

Granum, P.E., Whitaker, J.R. and Skjelkvale, R. (1981). Trypsin activation of enterotoxin from *Clostridium perfringens* type A. *Biochim. Biophys. Acta* **668,** 325–332.

Hanna, P.C., Mietzner, T.A., Schoolnik, G.K. and McClane, B.A. (1991). Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. *J. Biol. Chem*. **266,** 11037–11043.

Hanna, P.C., Wieckowski, E.U., Mietzner, T.A. and McClane, B.A. (1992). Mapping functional regions of *Clostridium perfringens* type A enterotoxin. *Infect. Immun*. **60,** 2110–2114.

Hanna, P.C., Wnek, A.P. and McClane, B.A. (1989). Molecular cloning of the 3′ half of the *Clostridium perfringens* enterotoxin gene and demonstration that this region encodes receptor-binding activity. *J. Bacteriol*. **171,** 6815–6820.

Hardy, S.P., Denmead, M., Parekh, N. and Granum, P.E. (1999). Cationic currents induced by *Clostridium perfringens* type A enterotoxin in human intestinal Caco-2 cells. *J. Med. Microbiol*. **48,** 235–243.

Hardy, S.P., Ritchie, C., Allen, M.C., Ashley, R.H. and Granum, P.E. (2001). *Clostridium perfringens* type A enterotoxin forms mepacrine-sensitive pores in pure phospholipid bilayers in the absence of putative receptor proteins. *Biochim. Biophys. Acta* **1515,** 38–43.

Horiguchi, Y., Akai, T. and Sakaguchi, G. (1987). Isolation and function of a *Clostridium perfringens* enterotoxin fragment. *Infect. Immun*. **55,** 2912–2915.

Horiguchi, Y., Uemura, T., Kozaki, S. and Sakaguchi, G. (1985). The relationship between cytotoxic effects and binding to mammalian cultures cells of *Clostridium perfringens* enterotoxin. *FEMS Microbiol. Let*. **28,** 131–135.

Huang, I.H., Waters, M., Grau, R.R. and Sarker, M.R. (2004). Disruption of the gene (*spoOA*) encoding sporulation transcription factor blocks endospore formation and enterotoxin productioin in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiol. Lett*. **233,** 233–240.

Katahira, J., Inoue, N., Horiguchi, Y., Matsuda, M. and Sugimoto, N. (1997a). Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. *J. Cell Biol*. **136,** 1239–1247.

Katahira, J., Sugiyama, H., Inoue, N., Horiguchi, Y., Matsuda, M. and Sugimoto, N. (1997b). *Clostridium perfringens* enterotoxin utilizes two structurally related membrane proteins as functional receptors *in vivo*. *J. Biol. Chem*. **272,** 26652–26658.

Kokai-Kun, J.F., Benton, K., Wieckowski, E.U. and McClane, B.A. (1999). Identification of a *Clostridium perfringens* enterotoxin

region required for large complex formation and cytotoxicity by random mutagenesis. *Infect. Immun*. **67,** 6534–6541.

Kokai-Kun, J.F. and McClane, B.A. (1997). Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infect. Immun*. **65,** 1014–1022.

Kominsky, S.L., Vali, M., Korz, D., Gabig, T.G., Weitzman, S.A., Argani, P. and Sukumar, S. (2004). *Clostridium perfringens* enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. *Am. J. Pathol*. **164,** 1627–1634.

Labbe, R.G. and Duncan, C.L. (1977). Evidence for stable messenger ribonucleic acid during sporulation and enterotoxin synthesis by *Clostridium perfringens* type A. *J. Bacteriol*. **129,** 843–849.

Long, H., Crean, C.D., Lee, W.H., Cummings, O.W. and Gabig, T.G. (2001). Expression of *Clostridium perfringens* enterotoxin receptors claudin-3 and claudin-4 in prostate cancer epithelium. *Cancer Res*. **61,** 7878–7881.

Majno, G. and Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol*. **146,** 3–15.

Marks, S., Kather, E., Kass, P. and Melli, A. (2002). Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. *J. Vet. Intern. Med*. **16,** 533–540.

Matsuda, M. and Sugimoto, N. (1979). Calcium-independent and calcium-dependent steps in action of *Clostridium perfringens* enterotoxin. *Biochem. Biophys. Res. Commun*. **91,** 629–636.

McClane, B.A. (1984). Osmotic stabilizers differentially inhibit permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. *Biochim. Biophys. Acta* **777,** 99–106.

McClane, B.A. (2000). *Clostridium perfringens* enterotoxin and intestinal tight junctions. *Trends Microbiol*. **8,** 145–146.

McClane, B.A. (2001). *Clostridium perfringens*. In: *Food Microbiology: Fundamentals and Frontiers*, (eds. M.P. Doyle, L.R. Beuchat, and T.J. Montville), pp. 351–372. ASM Press, Washington, D.C.

McClane, B.A. and McDonel, J.L. (1979). The effects of *Clostridium perfringens* enterotoxin on morphology, viability, and macromolecular synthesis. *J. Cell Physiol*. **99,** 191–200.

McClane, B.A. and McDonel, J.L. (1980). Characterization of membrane permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. *Biochim. Biophys. Acta*. **600,** 974–985.

McClane, B.A. and McDonel, J.L. (1981). Protective effects of osmotic stabilizers on morphological and permeability alterations induced in Vero cells by *Clostridium perfringens* enterotoxin. *Biochim. Biophys. Acta* **641,** 401–409.

McClane, B.A. and Wnek, A.P. (1990). Studies of *Clostridium perfringens* enterotoxin action at different temperatures demonstrate a correlation between complex formation and cytotoxicity. *Infect. Immun*. **58,** 3109–3115.

McDonel, J.L. (1986). Toxins of *Clostridium perfringens* types A, B, C, D, and E. In: *Pharmacology of Bacterial Toxins* (eds. F. Dorner and H. Drews), pp. 477–517. Pergamon Press, Oxford.

McDonel, J.L. and Demers, G.W. (1982). *In vivo* effects of enterotoxin from *Clostridium perfringens* type A in rabbit colon: binding vs. biologic activity. *J. Infect. Dis*. **145,** 490–494.

McDonel, J.L. and Duncan, C.L. (1975). Histopathological effect of *Clostridium perfringens* enterotoxin in the rabbit ileum. *Infect. Immun*. **12,** 1214–1218.

McDonel, J.L. and McClane, B.A. (1979). Binding vs. biological activity of *Clostridium perfringens* enterotoxin in Vero cells. *Biochem. Biophys. Res. Commun*. **87,** 497–504.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffen, P.M. and Tauxe, R.V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases* **5,** 607–625.

Melville, S.B., Collie, R.E. and McClane, B.A. (1997). Regulation of enterotoxin production in *Clostridium perfringens*. In: *The Molecular Genetics and Pathogenesis of the Clostridia* (eds. J.I. Rood, McClane, B.A., Songer, J.G. and Titball, R.), pp. 471–487 London, London.

Melville, S.B., Labbe, R. and Sonenshein, A.L. (1994). Expression from the *Clostridium perfringens cpe* promoter in *C. perfringens* and *Bacillus subtilus*. *Infect. Immun*. **62,** 5550–5558.

Michl, P., Buchholz, M., Rolke, M., Kunsch, S., Lohr, M., McClane, B., Tsukita, S., Leder, G., Adler, G. and Gress, T.M. (2001). Claudin-4: a new target for pancreatic cancer treatment using *Clostridium perfringens* enterotoxin. *Gastroenterology* **121,** 678–684.

Mietzner, T.A., Kokai-Kun, J.F., Hanna, P.C. and McClane, B.A. (1992). A conjugated synthetic peptide corresponding to the C-terminal region of *Clostridium perfringens* type A enterotoxin elicits an enterotoxin-neutralizing antibody response in mice. *Infect. Immun*. **60,** 3947–3951.

Miyamoto, K., Chakrabarti, G., Morino, Y. and McClane, B.A. (2002). Organization of the plasmid *cpe* locus of *Clostridium perfringens* type A isolates. *Infect. Immun*. **70,** 4261–4272.

Miyamoto, K., Wen, Q. and McClane, B.A. (2004). Multiplex PCR genotyping assay that distinguishes between isolates of *Clostridium perfringens* type A carrying a chromosomal enterotoxin gene (*cpe*) locus, a plasmid *cpe* locus with an IS*1470*-like sequence, or a plasmid *cpe* locus with an IS*1151* sequence. *J. Clin. Microbiol*. **41,** 1552–1558.

Sarker, M.R., Carman, R.J. and McClane, B.A. (1999). Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two *cpe*-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. *Molec. Microbiol*. **33,** 946–958.

Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K. and McClane, B.A. (2000). Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid versus chromosomal enterotoxin genes. *Appl. Environ. Microbiol*. **66,** 3234–3240.

Sherman, S., Klein, E. and McClane, B.A. (1994). *Clostridium perfringens* type A enterotoxin induces concurrent development of tissue damage and fluid accumulation in the rabbit ileum. *J. Diarrheal Dis. Res*. **12,** 200–207.

Singh, U., Mitic, L.L., Wieckowski, E., Anderson, J.M. and McClane, B.A. (2001). Comparative biochemical and immunochemical studies reveal differences in the effects of *Clostridium perfringens* enterotoxin on polarized CaCo-2 cells versus Vero cells. *J. Biol. Chem*. **276,** 33402–33412.

Singh, U., Van Itallie, C.M., Mitic, L.L., Anderson, J.M. and McClane, B.A. (2000). CaCo-2 cells treated with *Clostridium perfringens* enterotoxin form multiple large complex species, one of which contains the tight junction protein occludin. *J. Biol. Chem*. **275,** 18407–18417.

Smedley III, J.G. and McClane, B.A. (2004). Fine-mapping of the N-terminal cytotoxicity region of *Clostridium perfringens* enterotoxin by site-directed mutagenesis. *Infect. Immun*. 72:6914–6923

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Reviews* **9,** 216–234.

Sonoda, N., Furuse, M., Sasaki, H., Yonemura, S., Katahira, J., Horiguchi, Y. and Tsukita, S. (1999). *Clostridium perfringens* enterotoxin fragments remove specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J. Cell Biol*. **147,** 195–204.

Sparks, S.G., Carman, R.J., Sarker, M.R. and McClane, B.A. (2001). Genotyping of enterotoxigenic *Clostridium perfringens* isolates associated with gastrointestinal disease in North America. *J. Clin Microbiology* **39,** 883–888.

Varga, J., Stirewalt, V.L. and Melville, S.B. (2004). The CcpA protein is necessary for efficient sporulation and enterotoxin gene (*cpe*) regulation in *Clostridium perfingens. J. Bacteriol*. **186,** 5221–5229.

Wen, Q. and McClane, B.A. (2004). Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Appl. Environ. Microbiol*. **70,** 2685–2691.

Wen, Q., Miyamoto, K. and McClane, B.A. (2003). Development of a duplex PCR genotyping assay for distinguishing *Clostridium perfringens* type A isolates carrying chromosomal enterotoxin (*cpe*) genes from those carrying plasmid-borne enterotoxin (*cpe*) genes. *J. Clin. Microbiol*. **41,** 1494–1498.

Wieckowski, E., Kokai-Kun, J.F. and McClane, B.A. (1998). Characterization of membrane-associated *Clostridium perfringens* enterotoxin following Pronase treatment. *Infect. Immun*. **66,** 5897–5905.

Wieckowski, E.U., Wnek, A.P. and McClane, B.A. (1994). Evidence that an about 50kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically-bound *Clostridium perfringens* enterotoxin. *J. Biol. Chem*. **269,** 10838–10848.

Wnek, A.P., Strouse, R.J. and McClane, B.A. (1985). Production and characterization of monoclonal antibodies against *Clostridium perfringens* type A enterotoxin. *Infect. Immun*. **50,** 442–448.

Zhao, Y. and Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens. J Bacteriol* **180,** 136–142.

CHAPTER

# 46

# *Bacillus cereus* enterotoxins, bi- and tri-component cytolysins, and other hemolysins

*Nathalie Michelet, Per Einar Granum, and Jacques Mahillon*

## INTRODUCTION: *BACILLUS CEREUS SENSU LATO*

Whereas the virulence mechanisms of certain bacteria (*Salmonella, Shigella, Listeria*, or *Yersinia*) are in the process of being elucidated, our knowledge on the pathogenesis of other bacterial groups, including *Bacillus cereus sensu lato*, remains rather fragmentary. This chapter synthesizes our knowledge on the virulence factors found in this bacterial group. The specific toxins of each of the species constituting the *B. cereus* group are evoked in this chapter, but special attention is given to the generic virulence factors, widely distributed among the *B. cereus s.l.* species.

*B. cereus s.l.* is a group of six species of *Bacilliaceae*, displaying a large virulence spectrum. *B. anthracis*, the causative agent of anthrax, is highly monomorphic, non-mobile, non-hemolytic, and sensitive to penicillin. These phenotypic features are used to differentiate *B. anthracis* from the other members of the group (Turnbull, 1999). The other animal pathogen, *B. thuringiensis*, is active against larvae of lepidopteran, dipteran, and/or coleopteran insects. These two pathogenic organisms possess a series of virulence factors specific to their hosts. For *B. thuringiensis*, the major virulence factors are the Cry delta-endotoxins and/or Cyt cytolysins. They are produced during sporulation and form proteaneous crystals inside the sporange, a feature characteristic of this species. Upon ingestion by the insect larvae, the crystal is dissolved in the insect intestine and the protoxin is subsequently activated by the insect proteases. The free toxins specifically recognize and lyse the epithelial cells of the insect. This leads to a drop of the pH, germination of the bacterial spores, and the development of septicemia, which is frequently lethal (Schnepf *et al.*, 1998). In the case of *B. anthracis*, three proteins are necessary for the pathogen potential: the edema (EF) and lethal (LF) factors and the protective antigen (PA). The PA associates with one of the two others factors (EF or LF) to form the edema and the lethal toxins, respectively, leading to the specific syndrome of anthrax (Leppla, 1999).

*B. cereus sensu stricto* is an opportunistic bacterium known for its role in food infection and intoxication (see below), but it has also been found in other habitats (Jensen *et al.*, 2003), including the intestinal tract of arthropods, where it forms long filaments and leaves as symbiont. In this context, it is has been named *Arthromitus* (Margulis *et al.*, 1998). As indicated above, it is important to note that the only phenotypic trait used to differentiate *B. cereus* and the insect-associated *Arthromitus* from *B. thuringiensis* is the presence in the latter of the toxin crystals.

Recently, a new species, *B. weihenstephanensis*, has been proposed. This species is distinguished from the other *B. cereus* members by its psychrotolerant character and the genetic specificity of its 16S rDNA and heat shock protein *cspA* genes (Lechner *et al.*, 1998). However, these specific characteristics are still debated and do not seem to be unequivocal (Stenfors and

Granum, 2001). The two last members of the *B. cereus s.l.* group are *B. mycoides* and *B. pseudomycoides*. These species, characterized by their rhizoid growth on agar plates, can be distinguished by their fatty acid composition (Nakamura and Jackson, 1995; Nakamura, 1998). So far, no pathogenic or opportunistic activities have been associated with these bacteria.

In spite of their large virulence spectrum, members of the *B. cereus s.l.* share extremely close rDNA (Ash *et al.*, 1991; Ash and Collins, 1992; Daffonchio *et al.*, 2000) and could therefore be considered as the same species (Helgason *et al.*, 2000, 2004). Moreover, the genes coding for the entomopathogenic determinants of *B. thuringiensis* are on plasmids which can be exchanged among the different *B. cereus* members (Hu *et al.*, 2004), e. g., transforming a *B. cereus* in *B. thuringiensis* by plasmid acquisition (Thomas *et al.*, 2001). However, for simplicity and for socioeconomical reasons, it is preferable to maintain this identification system, in order to point out the specific virulence of each species.

## *BACILLUS CEREUS SENSU STRICTO*

*B. cereus s.s.*, the archetype of the *B. cereus s.l.* group, is frequently found as food toxi-infections, with symptoms of gastroenteritis (Granum, 1994). However, *B. cereus s.s.* is also a potential contaminant in hospitals because of the resistance of its spores to heat, radiation, and certain disinfectants, such as ethylic alcohol (Hsueh *et al.*, 1999). For these reasons, it is the causal agent of severe nosocomial infections in immunocompromised persons and may occasionally cause periodontitis, endocarditis, septicemia, meningitis, or pneumonia, generally after a surgical operation (Miller *et al.*, 1997; Castedo *et al.*, 1999; Gray *et al.*, 1999; Chu *et al.*, 2001; Gaur *et al.*, 2001). These cases are very rare, but the evolution of the disease is fulminating and often lethal. In each of these cases, the toxins involved are not yet precisely known. *B. cereus s.s.* is also associated with endophtalmitis, following a primary lesion. The contribution of the Hbl enterotoxin and/or phosphatidylcholine phospholipase (see below) has already been evoked. The effects of these toxins could be explained by the high proportion of phospholipids found in the retinal tissues (Beecher *et al.*, 2000; Beecher and Wong, 2000; Kotiranta *et al.*, 2000). However, after a phenotypic analysis of the corresponding *B. cereus* mutants, Callegan *et al.* could not point out differences between mutant and wild-type strains (Callegan *et al.*, 1999, 2002).

Foodstuffs potentially in contact with soil are particularly prone to *B. cereus s.s.* intoxication, for example, vegetables, fruits, milk, dehydrated milk, and spices or powdery products (Kotiranta *et al.*, 2000). The symptoms caused by *B. cereus s.s.* are divided in two types: the diarrheal and the emetic syndromes. The diarrheal syndrome was first described in Oslo in 1948, as the result of a vanilla sauce contamination in a hospital, while the emetic syndrome was noted some 20 years later following rice intoxication in London (Granum, 1997). However, the first case of food intoxication with acute gastroenteritis symptoms by *B. cereus* or a close bacterium was reported in 1906, when 300 persons in a hospital became ill after the ingestion of meatballs in which aerobic and sporulating bacteria were found (Kramer and Gibert, 1989).

The diarrheal syndromes appear 8 to 16 hours after ingestion of contaminated food, with an infective dose of between $10^5$ to $10^7$ CFU. They consist of abdominal pain, diarrhea, and often nausea and vomiting, which can last from 12 to 24 hours. For the emetic syndrome, the symptoms appear 0.5 to 5 hours after ingestion, and last from 6 to 24 hours (Granum and Lund, 1997). The emesis-causing dose is around 8 µg/kg in humans and in the house musk shrew (*Suncus murinus*) and around 10 µg/kg in the Rhesus monkey (Jaaskelainen *et al.*, 2003). The disease is characterized by nausea, vomiting, and malaise, and occasionally diarrhea (Granum and Lund, 1997). The causative agent of emesis is the cereulide, a dodecadepsipeptide synthesized by a non-ribosomal peptide synthetase, NRPS (Horwood *et al.*, 2004). It is a $K^+$-ionophoretic channel, highly resistant to pH between 2 to 11, to heat, and to proteolytic cleavage (Andersson *et al.*, 1998).

The number of intoxication cases by *B. cereus* is frequently underestimated because of the absence of severity in the symptoms. Since both types of syndromes are generally associated with particular food, their geographical repartition varies according to the feeding habits of the different regions of the world. For instance, there are 10 times more cases of emetic syndrome in Japan, whereas there is a majority of cases of diarrheal syndrome in Europe and North America (Kotiranta *et al.*, 2000).

Until now, two types of toxin detection kits have been used for the characterization of *B. cereus*. The BCET-RPLA kit (Oxoid) is based on the detection of the $L_2$ component of the HBL enterotoxin (see below) by inverse agglutination, while the TECRA kit from 3M reveals the 45k Da protein of the NHE enterotoxin by an ELISA sandwich test. However, in recent cases of food intoxication, *B. cereus* strains not expressing these two enterotoxins have been characterized (Lund *et al.*, 2000), indicating that other toxins, undetected by these methods, can play a role in the pathogenesis. Moreover, since the toxins detected by these kits are

also found in other *B. cereus s.l.* members, the detection specificity of these kits is not strictly limited to *B. cereus s.s*. Additional tests are therefore required for a more appropriate diagnostic (Guinebretière and Sanchis, 2003).

## TOXINS OF *B. CEREUS S.L.*

In addition to the specific virulence factors of *B. thuringiensis* and *B. anthracis*, most *B. cereus* group members possess secondary virulence factors involved in the invasion and/or survival of the bacteria in the host. Among these factors, some, for example, phospholipases, destroy the cell membrane by enzymatic activity while others, e.g., certain hemolysins, lyse the cells by pore formation. The virulence factors detailed in this section (Table 46.1) are the main secondary toxins for which experimental evidence has been reported or for which similarity exists with other known virulence factors.

### Phospholipases

Three types of phospholipases with different specificity have been studied in the *B. cereus s.l.* group. The sphingomyelinase (SM-PLC) cleaves sphingolipids, and the phosphatidylcholine phospholipase (PC-PLC) cleaves phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (Martin *et al.*, 2000). However, phosphatidylinositol phospholipase (PI-PLC) cleaves not only phosphatidylinositol, but also the derived glycolipids (Hondal *et al.*, 1998). As previously indicated, phospholipases cause cell lysis by enzymatic activity, unlike most hemolysins, which act by pore formation. The enzymatic reaction consists in a hydrolysis between the glycerol and the lipidic head.

#### *Sphingomyelinase (SM-PLC)*

The 999-nt sphingomyelinase gene encodes a mature protein of 306 aa residues preceded by a 27 residue signal peptide (Yamada *et al.*, 1988). In mammalian cells, the metabolites produced by the action of sphingomyelinases (ceramide, sphingomyeline, and sphingomyeline-1-phosphate) have an important function. They are involved in signal transduction, cellular proliferation, differentiation, and apoptosis (Obama *et al.*, 2003b; Yokomatsu *et al.*, 2003). The mammalian and bacterial enzymes both act at neutral pH and need $Mg^{+2}$ for activity. However, they present distinct sequences with some conserved residues that are present in the catalytic pocket of the enzyme. The mechanism of action of SM-PLC is deduced from its

**TABLE 46.1** Enterotoxins, cytolysins, and hemolysins from *B. cereus*

| Toxins | Genetic organization | Comments | References |
|---|---|---|---|
| Sphingomyelinase (SM-PLC) | *cerB* gene in the *cerAB* operon, regulated by PlcR | Component B of cereolysin AB | Yamada *et al.*, 1988 |
| Phosphatidylcholine phospholipase (PC-PLC) | *cerA* (*plcB*) gene in the *cerAB* operon, regulated by PlcR | Component A of cereolysin AB | Martin *et al.*, 2000 |
| Phosphatidylinositol phospholipase (PI-PLC) | *plcA* gene, regulated by PlcR | - | Kuppe *et al.*, 1989 |
| Hemolysin BL (Hbl) | *hblCDA* operon, coding for L2, L1 and B, respectively (ratio 1:1:1). Regulated by PlcR | B for binding, L1 and L2 are lytic components | Heinrichs *et al.*, 1993; Ryan *et al.*, 1997 |
| Non-hemolytic enterotoxin (Nhe) | *nheABC* operon, coding for NheABC, respectively (ratio 10:10: 1). Regulated by PlcR | NheC is a "catalyst" bringing together NheA and B. Similarities with the Hbl components | Granum *et al.*, 1999; Lindbäck *et al.*, 2004 |
| Cytotoxin K (Cyt K) | *cytK* gene, regulated by PlcR | Two forms exist: Cyt K-1 and Cyt K-2. They belong to the "β-barrel pore-forming toxin" family | Lund *et al.*, 2000; Fagerlund *et al.*, 2004 |
| Hemolysin II (Hly-II) | *hly-II* gene | Belongs to the "β-barrel pore-forming toxin" family | Baida *et al.*, 1999 |
| Hemolysin III (Hly-III) | *hly-III* gene | - | Baida and Kuzmin, 1996 |
| Cereolysin O (Clo) | *clo* gene, regulated by PlcR | Belongs to the "cholesterol-binding cytolysin" family. Synomyns are thuringiolysin O (TLO) and anthrolysin O (ALO) | Shany *et al.*, 1974 |

similarity to DNase I. The His296 residue (general base) first activates a water molecule, which, in turn, attacks the phosphodiester bond of sphingomyeline. The water molecule could interact not only with the His296, but also with the essential $Mg^{+2}$. This attack results in an electron transfer to His151 (general acid), by a penta-covalent intermediate, stabilized by $Mg^{+2}$, and the reaction ends by the liberation of ceramide (Obama *et al*., 2003a). Another essential residue is Asp195, which is thought to maintain the special arrangement of this acid-base catalyst. The His-Asp-His triad seems to be important because it is also found in the PI-PLC, which belongs to another enzyme family (Obama *et al*., 2003a).

***Phosphatydylcholine phospholipase (PC-PLC)***

The PC-PLC gene lies upstream of that of SM-PLC. This 245-aa $Zn^{+2}$ metalloprotein (28.5 kDa) provides the bacteria with a lecithinase activity but not a hemolytic activity. However, SM-PLC and PC-PLC can form an effective hemolytic complex, named cereolysin AB (CerAB). It is important to note that the α-toxin of *Clostridium perfringens* groups both activities in one gene coding for a 43-kDa protein. This observation suggests that either the intergenic space present in *B. cereus* has disappeared in *C. perfringens* or, conversely, that an intervening sequence has been inserted between the *B. cereus* genes (Gilmore *et al*., 1989). In mammalian cells, the diacylglycerol, one of the products formed by PC-PLC action, acts as a second messenger in signal transduction cascade, serving as an endogenous activator of protein kinase C (Exton, 1997). The *B. cereus* PC-PLC mimics this function when applied to eukaryotic cells (Diaz-Laviada *et al*., 1990), and expression of this toxin in fibroblasts leads to oncogenic transformation of the cells (Johansen *et al*., 1994).

The Glu4, Tyr56, and Phe66 residues of PC-PLC are present in the catalytic pocket, but are not directly involved in the catalytic process. They are components of the headgroup binding site. Mutagenesis experiments have suggested that these residues play a role in modulating substrate specificity of the enzyme. There is evidently a direct interaction between the aromatic ring of Phe66 and the substrate headgroup, especially choline and ethanolamine, since replacing this residue by another residue without an aromatic ring reduces the activity of the enzyme. The charge of residue Glu4 is important because a negatively charged residue increases choline and ethanolamine specificity, whereas a polar neutral or positively charged residue increases serine specificity. The presence of an aromatic residue at position 56 confers preference for a substrate with a positive headgroup (Antikainen *et al*., 2003).

***Phosphatydylinositol phospholipase (PI-PLC)***

The phosphatidylinositol phospholipase is a ubiquitous protein found in both eukaryotic and prokaryotic organisms. Indeed, in eukaryotes, degradation products of PI-PLC (diacyglycerol and inositol triphosphate) are signaling molecules, important in metabolic pathways (Gassler *et al*., 1997), such as activation of protein kinase C or intracellular calcium mobilization. The PI-PLC of *B. cereus* is frequently used as a model because it is smaller than its eukaryotic equivalent (Kuppe *et al*., 1989). However, the bacterial enzyme only accepts non-phosphorylated phosphatydylinositol and produces mainly cyclic inositol phosphate, whereas the mammalian enzyme prefers phosphorylated substrate and produces cyclic and non-cyclic inositol. Both proteins act by a general acid-base mechanism, but the mammalian enzyme uses $Ca^{+2}$ while the *B. cereus* PI-PLC does not use ions. In the active pocket, the location of the $Ca^{+2}$ is replaced by an arginine side chain. This residue stabilizes the transition state of the phosphoryl transfer reaction and facilitates the deprotonation of the 2-OH from the inositol ring (Kravchuk *et al*., 2003). Three residues (His82-Asp33-Arg69) are involved in the activation of the phosphate group and protonate the leaving group in a highly cooperative manner. The dyad Asp274-His32 serves as a general base. The H-bond between these two residues is a short strong hydrogen bond. This type of binding provides the stabilization energy needed for an intermediate or a transition state, as frequently seen in general acid-base catalytic mechanisms (Zhao *et al*., 2004).

The bacterial enzyme cleaves phosphatydylinositol in a rapid intramolecular transphosphorylation reaction to form diacylglycerol and D-myo-inositol-1,2-cyclic phosphate. In a second reaction, the cyclic phosphorylase activity of PI-PLC catalyzes the slow hydrolysis of the cyclic phosphate to D-myo-inositol-1-phosphate (Birrell *et al*., 2003). The enzyme activity of the first reaction is enhanced by non-substrate lipids, as phosphatidylcholine. The catalytic efficiency of the second step is enhanced by the addition of a water-soluble organic solvent, such as isopropanol, dimethylsulfoxide, and dimethylformamide and by phosphatidylcholine (Birrell *et al*., 2003; Wehbi *et al*., 2003).

Helix 42-48 and loop 237-243 of PI-PLC surround the rim of the catalytic pocket and contain a high number of hydrophobic residues exposed to the solvent. These residues penetrate in the lipid bilayer and stabilize the membrane-protein complex. Therefore, the membrane fluidity is important for the enzyme activity because the hydrophobic residues can penetrate more easily in the membrane bilayer and the complex is also more stable (Lehto and Sharom, 2002). The region at the top of the barrel rim contains two tryptophans

exposed to the solvent, which seem essential for protein binding to phosphatidylcholine and for the enzyme kinetic activation. The high number of acidic and basic residues at the surface of PI-PLC suggests the formation of enzyme aggregates. One mechanism for interfacial activation is the oligomerization of the enzyme at the lipid surface, providing the aggregate a higher activity than monomers (Zhang *et al*., 2004). So the raft formation allows a higher local concentration of substrate and increases the enzyme activity. The enzyme stays bound to the membrane for multiple catalytic turnover cycles (Lehto and Sharom, 2002).

The PI-PLC is a small enzyme of 35 kDa and 298 amino acid residues, monomeric with one active site and one subsite, which is an activator site causing an activity increase. So two substrate molecules can bind simultaneously to the enzyme. This is compatible with the interfacial activation model—the binding of the first phospholipids represents the initial step in the process that may lead to interfacial activation (Birrell *et al*., 2003). Finally, note that the PI-PLC gene is not grouped with the other two phospholipase genes and does not share similarity with them (Kuppe *et al*., 1989).

## Enterotoxins

*B. cereus* produces at least three different cytotoxins that can be involved in food poisoning (Table 46.1). The three toxins are hemolysin BL (Beecher and Macmillan, 1991; Heinrichs *et al*., 1993; Ryan *et al*., 1997), non-hemolytic enterotoxin (Lund and Granum, 1996; Granum *et al*., 1999), and Cytotoxin K (Lund *et al*., 2000). The suggested enterotoxin BceT (Agata *et al*., 1995) has just been shown to be a gene fusion product between a genomic *B. cereus* gene and a gene with homology to ORF101 in *B. anthracis* pXO1 (Hansen *et al*., 2003). Nothing is known about the role of enterotoxin FM in food poisoning, a "toxin" that has only been cloned without any biological characterization (Asano *et al*., 1997). However, enterotoxin FM has sequence homology to a cell wall hydrolase from *Bacillus subtilis* (Margot *et al*., 1998), and is probably not enterotoxic at all. The two latter proposed "toxins" are therefore not treated in this chapter.

Substantial work has been carried out on the occurrence of the three different enterotoxins in strains of *B. cereus*, and almost all strains (about 99%) contain *nhe*, about 50% contain *hbl*, and around 40% contain *cytK* (EU-project QLK-CT-2001-00854). There are two forms of CytK (Fagerlund *et al*., 2004) and only two strains have so far been found to harbor the original *cytK-1* gene (Granum *et al*., unpublished results). A mixture of the three toxins described above has been shown to not survive the stomach acid and proteolytic enzymes in the duodenum (Granum *et al*., 1993). Thus it seems likely that only when the toxins are produced and secreted in the small intestine during vegetative growth are they able to cause diarrhea, which is also supported by the fact that the incubation time is usually greater than eight hours (Granum, 2001).

All three enterotoxins from the *B. cereus* group are cytotoxic and cell membrane active toxins that will make channels or holes in the membranes (Granum, 2001). At high concentration, the membrane system will disintegrate.

### *Hemolysin BL (Hbl)*

Two of the three characterized enterotoxins from *B. cereus* have three components. The three-component hemolysin (Hbl; consisting of the B, L1, and L2 proteins) with enterotoxin activity was the first to be fully characterized (Beecher and Wong, 1994; Beecher and Wong, 1997). This toxin also has dermonecrotic and vascular permeability activities, and causes fluid accumulation in ligated rabbit ileal loops (Beecher *et al*., 1995). Although Hbl has been suggested to be a primary virulence factor in *B. cereus* diarrhea (Beecher *et al*., 1995), it should be stressed that about half of the food poisoning strains do not produce this enterotoxin (Guinebretière *et al*., 2002; Stenfors *et al*., 2002). It is also less toxic on epithelial cells than Nhe (Lund and Granum, 1997; Lindbäck *et al*., 2004). It has been shown that all three components are necessary for maximal enterotoxin activity (Beecher *et al*., 1995). It was first suggested that the B protein is the component that binds the Hbl complex to the target cells, and that L1 and L2 have lytic functions (Beecher and Macmillan, 1991). However, more recently another model for the action of Hbl has been proposed, suggesting that the components of Hbl bind to target cells independently and then constitute a membrane-attacking complex, resulting in a colloid osmotic lysis mechanism (Beecher and Wong, 1997). A 1:1:1 ratio of the three components seems to give the highest biological activity (Beecher *et al*., 1995). Substantial heterogeneity has been observed in the Hbl components, and individual strains produce various combinations of single or multiple bands of each component (Schoeni and Wong, 1999). This could be due to multiple genes of *hbl* with sequence variation, but this still needs to be established genetically.

The organization of the *hbl* operon is given in Figure 46.1. The reason for not starting with *hblA* is that the operon was cloned and sequenced in two different laboratories (Heinrichs *et al*., 1993; Ryan *et al*., 1997), and the sequence of the B-component (hblA) was published first. The proteins seem to be produced in close to a 1:1:1 ratio as judged from 2D gel studies (Gohar

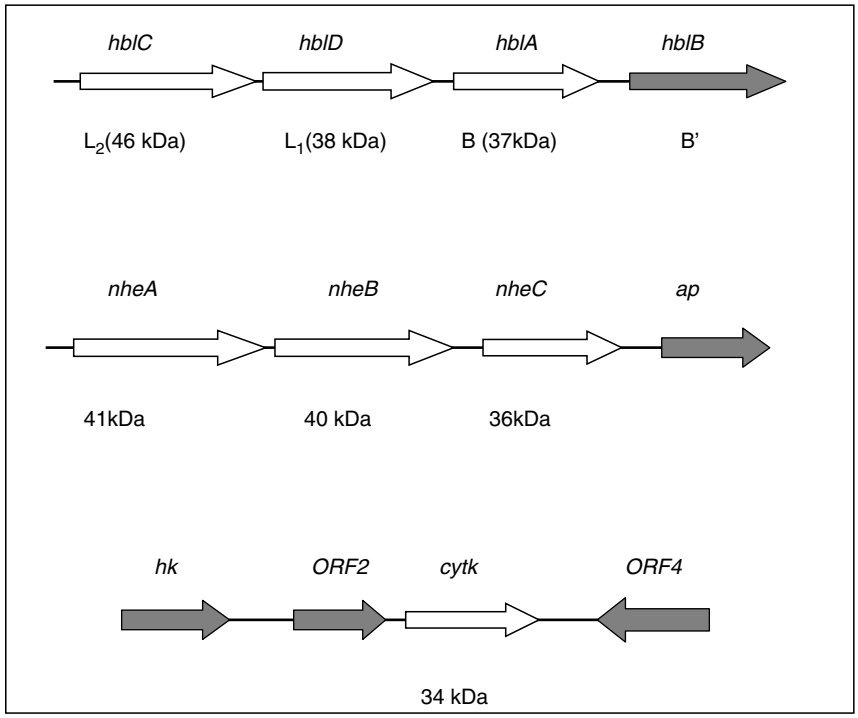



**FIGURE 46.1** Genetic organization of the three enterotoxins: *hbl*, *nhe*, and *cytK* (based on Heinrichs *et al*., 1993; Ryan *et al*., 1997; Granum *et al*., 1999; and Lund *et al*., 2000). The white arrows refer to the genes encoding the enterotoxins. There is an inverted repeat of 13 bp between *nheB* and *nheC*. An aminopeptidase gene (*ap*) is found about 600 bp downstream of *nheC*. *CytK* has an open reading frame (*ORF2*) upstream, encoding a protein of unknown function, and a histidine kinase (*hk*) further upstream. *ORF4* encodes for a long-chain-fatty-acid-CoA ligase (orientated in opposite direction to that of *cytK*). The arrowheads indicate the orientation of the genes. The name of the genes, proteins, and size of the toxin components are also indicated.

*et al*., 2002). HblB' is similar to hblB but its actual function is not known, and it is not necessary for biological activity (Lund and Granum, 1997). As the name suggests, the Hbl tri-component enterotoxin is also hemolytic, and since many papers have dealt with the properties of this protein complex, it will not be treated here.

### *The non-hemolytic enterotoxin (Nhe)*

A non-hemolytic, three-component enterotoxin (Nhe) was characterized after an outbreak involving 152 people caused by an *hbl* negative strain (Lund and Granum, 1996). These three components were different from those of Hbl, although there are similarities. A combination of the NheA and NheB components of this enterotoxin possess some biological activity after purification, but this is due to minor contamination with NheC that will always co-purify with the two other proteins (primarily with NheB) encoded by this operon (Figure 46.1) (Lund and Granum, 1997; Lindbäck *et al*., 2004). The optimal ratio between the NheA, NheB, and NheC components is 10:10:1, respectively (Lindbäk *et al*., 2004). It is also clear that it is not possible to find NheC alone in cell extracts after growth of *B. cereus* (Gohar *et al*., 2002; Lindbäck *et al*., 2004). Therefore, recombinant proteins have been used to establish the ratio between the different components (Lindbäck *et al*., 2004). An important observation is that a concentration of NheC higher than about 10% of that of NheA and NheB inhibited the toxic activity of Nhe (Lindbäck *et al*., 2004). All *B. cereus* strains seem to have an inverted repeat of 13 bp between *nheB* and *nheC* that probably contributes to (or determines) the transcribed/translated ratio between the three components. In some strains, even more complex repeated sequences have been recorded (Sekse and Granum: MSc thesis 2002, Norwegian Agriculture University). Such structures may either stabilize the mRNA upstream of *nheC* or reduce the efficiency of translational initiation since it is strongly correlated with the structure of the RBS (Schlax and Worhunsky, 2003).

Results from cell-binding experiments followed by Western immunoblotting have shown that NheB was the only individual component that bound to the Vero cells. Neither NheA nor NheC could be observed attached to the Vero cells. Also, if too much NheC is added to the reaction mixture on Vero cells, the cytotoxicity is reduced (Lindbäck *et al*., 2004). It looks as though NheC binds to NheB and prevents NheB from binding to receptors on the epithelial cells, thus reducing the total activity of the toxin complex (Lindbäck *et al*., 2004). Although we have no proof of the function of NheC, it seems likely that it functions as a "catalyst," either by bringing NheA and NheB together (after binding of NheB to the target cells) or by enhancing conformational changes to the complex components.

Although there are structural and sequence similarities between Hbl and Nhe, components from one complex cannot substitute for components from the other (Lund and Granum, 1997). The identity is highest in

the N-terminal third of the proteins. The most pronounced similarities are found between *nheA* and *hblC*, *nheB* and *hblD*, and *nheC* and *hblA*. This is not only in direct comparison of the sequences, but also in predicted transmembrane helices for the six proteins. NheA and HblC have no predicted transmembrane helices, while NheB and HblD have two each. Finally, NheC and HblA each have one predicted transmembrane helix in the same relative position (Granum *et al*., 1999).

Different types of experiments have indicated that Nhe contribute to about 80% of the cytotoxicity in strains of *B. cereus* (Märtelbauer *et al*., unpublished results). This is partly due to the difference in toxicity per weight unit (100 ng of Nhe is more toxic than 100 ng of Hbl), and partly due to differences in the production and secretion of the proteins.

### *Cytotoxin K (CytK)*

The newly discovered enterotoxin cytotoxin K (CytK), similar to the β-toxin of *C. perfringens* (and other related toxins), was the cause of the symptoms in a severe outbreak of *B. cereus* food-borne illness in France in 1998 (Lund *et al*., 2000). In this outbreak several people developed bloody diarrhea and three died. It would be fair to call this an outbreak of *B. cereus* necrotic enteritis, although it is not nearly as severe as the *C. perfringens* type C food poisoning (Brynestad and Granum, 2002). The amino acid sequence of CytK is similar (about 30% identity) to that of α-hemolysin (α-toxin, α-HL), leucocidins, and γ-hemolysin of *Staphylococcus aureus* and β-toxin of *C. perfringens*, which all belong to the family of β-barrel pore-forming toxins (Song *et al*., 1996; Prevost, 1999; Steinthorsdottir *et al*., 2000). The capacity to form pores in planar bilayers (Hardy *et al*., 2001) is consistent with CytK being a member of this family of proteins with pore-forming ability. It has been shown to be weakly anion selective and to exhibit an open channel probability close to one (Hardy *et al*., 2001). The predicted minimum pore diameter is approximately 7Å. CytK, like other β-barrel pore-forming toxins, spontaneously forms oligomers (heptamers), which are resistant to SDS, but not to boiling. These heptamers are able to form pores in planar bilayers and to kill erythrocytes, but have no cytotoxicity on epithelial cells (Fagerlund *et al*., 2004).

Two forms of CytK have been characterized, the original CytK (Figure 46.1; renamed CytK-1) and CytK-2 with a deduced amino acid sequence 89% identical to that of the original CytK-1. There are only marginal differences among variants of the *cytK-2* (97–99% identity in amino acid sequences) found in the majority of *B. cereus* strains (Fagerlund *et al*., 2004). Only two strains out of several hundred have the original *cytK-1* gene, and it is also organized in an identical manner on the chromosome/plasmid (P.E. Granum, unpublished results). CytK-1 is about five times more toxic on epithelial cells than CytK-2, but the activity on erythrocytes seems to be very similar. In the strain that killed three people in France, CytK-1 was the only protein present with cytotoxic activity towards epithelial cells. It is therefore tempting to speculate that CytK-1 forms ulcerative and hemorrhagic lesions in the intestine when secreted alone without the other two enterotoxins that result in watery diarrhea. Several strains that produce CytK-2 have also been reported to cause food poisoning without causing bloody diarrhea (Fagerlund *et al*., 2004).

The CytK-2 proteins show relatively high sequence similarity (39% identity) to the deduced product of *hly-II* (Baida *et al*., 1999). However, in aligning the deduced sequences of *cytK* and *hly-II*, 6% gaps are present, whereas none are observed when comparing CytK-1 and the CytK-2 proteins. Also, Hly-II is unlikely to contribute to food poisoning since there is a lysine residue at the end of the β-barrel loop penetrating the cell membrane. This will readily be cut by trypsin in the small intestine, and inactivate the toxin as for the *C. perfringens* β-toxin (Fagerlund *et al*., 2004).

## Hemolysins

### *Cereolysin AB (CerAB)*

As mentioned above, phospholipases PC-PLC and SM-PLC can act together to induce hemolytic activity, e.g., against human erythrocytes (Gilmore *et al*., 1989). This complex is named cereolysin AB.

### *Hemolysin II (Hly-II)*

The 412-residue Hly-II protein (45.6 kDa and 42.3 kDa without its signal peptide) displays about 30% identity with a toxin family called β-barrel pore-forming toxins, with α-hemolysin of *S. aureus* or CytK of *B. cereus* (see above) (Sinev *et al*., 1993; Baida *et al*., 1999). This family of heptameric toxins is characterized by two anti-parallel transmembrane and glycin-rich strands (Menestrina *et al*., 2001). Hly-II possesses 94 additional amino acids, in comparison with the other members of the toxin family. These residues do not seem to be essential to the activity, and the addition of this tail to the other toxins of the family does not cause problems. However, the specific activity of hemolysin II seems higher than that of the other β-barrel pore-forming toxins (Baida *et al*., 1999; Miles *et al*., 2002). The hemolysin II displays an important Arrhenius effect (regain of activity after a short increase of temperature up to 90–100°C), and a long lag period before appearance of hemolysis in the activity tests with blood-containing media (Sinev *et al*.,

1993). Distribution studies of the toxin in *B. cereus* and *B. thuringiensis* suggest that the toxin is most frequently found in *B. thuringiensis*, but its distribution remains limited (Budarina *et al*., 1994).

### *Hemolysin III (Hly-III)*

Hemolysin 3 is the least characterized hemolytic toxin from the *B. cereus* group. Its *hly-III* gene, cloned and characterized in *Escherichia coli*, is 657 nucleotides long and encodes a product with a calculated molecular mass of 24.4 kDa (Baida and Kuzmin, 1995). The toxin acts by oligomeric pore formation in three steps: the protein first binds to the erythrocyte surface, monomers are then assembled to form the transmembrane pore, leading to erythrocyte lysis. While the first two steps are temperature dependent, the final lysis is not (Baida and Kuzmin, 1996).

### *Cereolysin O (CLO)*

The cereolysin O (CLO, also named thuringiolysin O in *B. thuringiensis*, or anthrolysin O in *B. anthracis*) (Shannon *et al*., 2003) is a member of the cholesterol-binding cytolysins (CBC). This family groups cytolysins of certain pathogens, such as *Streptococcus pyogenes* (streptolysin O), *C. perfringens* (perfringolysin O), or *Listeria monocytogenes* (listeriolysin O). As the name indicates, they bind to cholesterol at the cell surface (Shany *et al*., 1974). They are inactivated by oxidation, but the addition of a reducing agent restores the activity. Most of the toxin family members possess one cysteine located in the most conserved, 11-aa domain of the protein (ECTGLAWEWWR). However, this cysteine does not seem to be essential to the toxin activity, whereas the tryptophan residues seem absolutely necessary. They are needed for the membrane insertion with their large hydrophobic core. The pore formed by CBC is more than 150 Å, which is about 50 monomers and permits the passage of large molecules (Palmer, 2001). Distribution studies on the cereolysin O gene have shown its ubiquity in all the *B. cereus s.l.* members. Moreover, the gene seems to be much conserved (more than 95% of identity) and does not differentiate the six species. It is also suggested that the gene is present in more than one copy in some strains (Michelet and Mahillon, unpublished results).

## REGULATION OF VIRULENCE FACTORS IN *B. CEREUS S.L.*

What do we know about the regulation of virulence genes of *B. cereus s.l. ?* One central pleiotropic transcriptional regulator (PlcR) has been characterized in the *B. cereus s.l.* by the group of Lereclus and colleagues. (Lereclus *et al*., 1996; 2000). It regulates the expression of a large number of virulence-related genes (possibly more than 100), among which some have been confirmed experimentally: *plcA* (PI-PLC), *plcB* (PC-PLC), *cerB* (SM-PLC), *hblCDA* (Hbl), *nheABC* (Nhe), *cytK*, *clo*, *colB* (collagenase), *sfp* (protease), and three metalloprotease genes *inhA2*, *nprB*, and *nprP2*.

It was shown that inactivation of the *plcR* gene caused drastic diminution of the pathogenic potential of *B. cereus* and *B. thuringiensis* in mice and insects (Salamitou *et al*., 2000). All the PlcR-regulated genes possess a palindromic consensus sequence (TATG-NANNNNTNCATA) found from 35 to 250 nucleotides before transcription starts (Agaisse *et al*., 1999). Some divergences of this palindromic sequence can arise without drastically affecting the PlcR activation. It is the case for the *cytK* gene (Brillard and Lereclus, 2004) and for *inhA2* encoding the InhA2 metalloprotease (Fedhila *et al*., 2003).

PlcR regulates its own expression, and downstream of the PlcR gene is a short ORF of 48 amino acids. This peptide (PapR for peptide activating PlcR) is secreted by the bacteria, processed extracellularly in a pentapeptide, and reimported into the cell by an oligopeptide permeation system (Opp A, B, C, D, and E), which is essential for PlcR regulation. PapR binds PlcR and promotes the fixation of the molecule to DNA. The regulation is controlled by the bacterial concentration (quorum sensing). In growth phase, low concentration of PapR does not allow the expression of PlcR regulon, while at the start of stationary phase, concentration increases and the *plcR* transcription is activated (Lereclus *et al*., 1996; Gominet *et al*., 2001; Slamti and Lereclus, 2002). An additional regulation mechanism involves Spo0A. Two fixation sites of Spo0A are found upstream from the *plcR* gene. In sporulation medium, Spo0A is activated and binds DNA, inhibiting the expression of *plcR*. So, in poor medium, *plcR* is blocked and virulence genes are not expressed. In rich medium, the transitory phase is long, and the expression of *plcR* is effective during this period, before the activation of Spo0A (Lereclus *et al*., 2000).

*B. anthracis* possesses an arsenal of virulence genes similar to those found in the other members of the *B. cereus* group. Yet, *B. anthracis* has a truncated *plcR* gene (Agaisse *et al*., 1999). So the PlcR regulon is not expressed, but it can be reactivated by the incorporation of active PlcR (Mignot *et al*., 2001). However, Pomerantsev *et al*., have constructed a plasmid with PlcR-PapR that did not activate the hemolytic genes (Pomerantsev *et al*., 2003). Conversely, a plasmid harboring a deletion in the PlcR and PapR intergenic region induced hemolytic activity without the PapR

secretion, processing, and reimportation (Pomerantsev *et al*., 2004).

Recently, the *B. anthracis* hemolytic genes were induced by strictly anaerobic conditions, suggesting an alternative regulatory mechanism for the expression of virulence genes (Klichko *et al*., 2003). It also seems that activating factors may be found in Brain-Heart-Infusion medium because hemolytic activity was found for *B. anthracis* growing in this medium compared to LB medium (Shannon *et al*., 2003). An alternative activation pathway is also suggested by the recent work on cereolysin O, which does not seem to follow the PlcR expression timing (Michelet and Mahillon, unpublished results).

Finally, an interesting *B. thuringiensis* mutant defective in swimming and swarming has been characterized. It lacked the peritrichous flagella found in the *B. cereus s.l.* family. This mutant is interesting because the absence of flagella was correlated with the absence of secretion of the Hbl and PC-PLC toxins. In fact, it is a mutation in the *flhA* gene that causes this phenotype. FlhA is involved in the export of structural protein of the flagellum. In the mutant, the flagella protein, as well as the Hbl and PC-PLC proteins, were expressed but not secreted. However, the thuringiolysin/cereolysin O, Hly-II, and Hly-III were apparently produced normally by these mutants (Ghelardi *et al*., 2002). Thus, the exact interconnections among these regulatory circuits (motility versus virulence) remain to be elucidated.

## CONCLUSION

The species belonging to the *B. cereus s.l.* group are genetically closely related (their 16S rRNA are almost identical), and the generic virulence determinants are largely distributed among them. Yet, they display a large spectrum of pathogenesis. Until now, the particular contribution of each of these virulence factors was not well understood. Which of them are involved in the different types of pathogenesis? Do they act independently or synergistically? Why do some species possess virulence genes without exploiting them, at least apparently? Are they non-functional or potentially active under certain conditions? These questions are particularly relevant because of the presence, in this group, of key bacteria. It is the case for *B. thuringiensis*, which is the most important biopesticide used for several decades in the control of insect pests or vector of human and animal diseases. Is this bacterium a potential human virulent agent?

*B. anthracis* represents another important issue because of the severity of the disease it causes. Although the inactivation of its transcriptional regulator PlcR has now been well documented, alternative regulatory pathways have been suggested. Do the other bacteria of the group possess these alternative solutions for virulence gene expression? Determination of the complete genome sequences of several *B. cereus s.l.* will certainly permit us to point out the diversity of the virulent genes and their regulatory sequences. Ultimately, however, the only way to unravel the precise role(s) of the toxins in pathogenesis will be to study mutants inactivated for (or strains lacking) individual or combinations of virulence factors.

## REFERENCES


Agaisse, H., Gominet, M., Okstad, O.A., Kolstø, A.B. and Lereclus, D. (1999). PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis. Mol. Microbiol*. **32,** 1043–1053.

Agata, N., Ohta, M., Arakawa, Y. and Mori, M. (1995). The *bceT* gene of *Bacillus cereus* encodes an enterotoxic protein. *Microbiology* **141,** 983–988.

Andersson, M.A., Mikkola, R., Helin, J., Andersson, M.C. and Salkinoja-Salonen, M. (1998). A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. *Appl. Environ. Microbiol*. **64,** 1338–1343.

Antikainen, N.M., Hergenrother, P.J., Harris, M.M., Corbett, W. and Martin, S.F. (2003). Altering substrate specificity of phosphatidylcholine-preferring phospholipase C of *Bacillus cereus* by random mutagenesis of the headgroup binding site. *Biochemistry* **42,** 1603–1610.

Asano, S.I., Nukumizu, Y., Bando, H., Iizuka, T. and Yamamoto, T. (1997). Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis. Appl. Environ. Microbiol*. **63,** 1054–1057.

Ash, C. and Collins, M.D. (1992). Comparative analysis of 23S ribosomal RNA gene sequences of *Bacillus anthracis* and emetic *Bacillus cereus* determined by PCR-direct sequencing. *FEMS Microbiol. Lett*. **73,** 75–80.

Ash, C., Farrow, J.A., Dorsch, M., Stackebrandt, E. and Collins, M.D. (1991). Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int. J. System. Bacteriol*. **41,** 343–346.

Baida, G., Budarina, Z.I., Kuzmin, N.P. and Solonin, A.S. (1999). Complete nucleotide sequence and molecular characterization of hemolysin II gene from *Bacillus cereus. FEMS Microbiol. Lett*. **180,** 7–14.

Baida, G.E. and Kuzmin, N.P. (1995). Cloning and primary structure of a new hemolysin gene from *Bacillus cereus. Biochim. Biophys. Acta*. **1264,** 151–154.

Baida, G.E. and Kuzmin, N.P. (1996). Mechanism of action of hemolysin III from *Bacillus cereus. Biochim. Biophys. Acta* **1284,** 122–124.

Beecher, D.J. and Macmillan, J.D. (1991). Characterization of the components of hemolysin BL from *Bacillus cereus*. *Infect. Immun*. **59,** 1778–1784.

Beecher, D.J., Olsen, T.W., Somers, E.B. and Wong, A.C. (2000). Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. *Infect. Immun*. **68,** 5269–5276.

Beecher, D.J., Schoeni, J.L. and Wong, A.C. (1995). Enterotoxic activity of hemolysin BL from *Bacillus cereus. Infect. Immun*. **63,** 4423–4428.

Beecher, D.J. and Wong, A.C. (1994). Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus. Infect. Immun*. **62,** 980–986.

Beecher, D.J. and Wong, A.C. (1997). Tripartite hemolysin BL from *Bacillus cereus*. Hemolytic analysis of component interactions and a model for its characteristic paradoxical zone phenomenon. *J. Biol. Chem*. **272,** 233–239.

Beecher, D.J. and Wong, A.C. (2000). Tripartite hemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. *Microbiology* **146,** 1371–1380.

Birrell, G.B., Zaikova, T.O., Rukavishnikov, A.V., Keana, J.F. and Griffith, O.H. (2003). Allosteric interactions within subsites of a monomeric enzyme: kinetics of fluorogenic substrates of PI-specific phospholipase C. *Biophys. J*. **84,** 3264–3275.

Brillard, J. and Lereclus, D. (2004). Comparison of cytotoxin CytK promoters from *Bacillus cereus* strain ATCC 14579 and from a *B. cereus* food-poisoning strain. *Microbiology* **150,** 2699–2705.

Brynestad, S. and Granum, P.E. (2002). *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol*. **74,** 195–202.

Budarina, Z.I., Sinev, M.A., Mayorov, S.G., Tomashevski, A.Y., Shmelev, I.V. and Kuzmin, N.P. (1994). Hemolysin II is more characteristic of *Bacillus thuringiensis* than *Bacillus cereus. Arch. Microbiol*. **161,** 252–257.

Callegan, M.C., Cochran, D.C., Kane, S.T., Gilmore, M.S., Gominet, M. and Lereclus, D. (2002). Contribution of membrane-damaging toxins to *Bacillus* endophthalmitis pathogenesis. *Infect. Immun*. **70,** 5381–5389.

Callegan, M.C., Jett, B.D., Hancock, L.E. and Gilmore, M.S. (1999). Role of hemolysin BL in the pathogenesis of extraintestinal *Bacillus cereus* infection assessed in an endophthalmitis model. *Infect. Immun*. **67,** 3357–3366.

Castedo, E., Castro, A., Martin, P., Roda, J. and Montero, C.G. (1999). *Bacillus cereus* prosthetic valve endocarditis. *Ann. Thorac. Surg*. **68,** 2351–2352.

Chu, W.P., Que, T.L., Lee, W.K. and Wong, S.N. (2001). Meningoencephalitis caused by *Bacillus cereus* in a neonate. *Hong Kong Med. J*. **7,** 89–92.

Daffonchio, D., Cherif, A. and Borin, S. (2000). Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group." *Appl. Environ. Microbiol*. **66,** 5460–5468.

Diaz-Laviada, I., Larrodera, P., Diaz-Meco, M.T., Cornet, M.E., Guddal, P.H., Johansen, T. and Moscat, J. (1990). Evidence for a role of phosphatidylcholine-hydrolyzing phospholipase C in the regulation of protein kinase C by *ras* and *src* oncogenes. *EMBO J*. **9,** 3907–3912.

Exton, J.H. (1997). Cell signaling through guanine nucleotide-binding regulatory proteins (G proteins) and phospholipases. *Eur. J. Biochem*. **243,** 10–20.

Fagerlund, A., Ween, O., Lund, T., Hardy, S. P. and Granum, P. E. (2004). Different cytotoxicity of CytK and CytK-like proteins from *B. cereus. Microbiology* **150,** 2689–2697.

Fedhila, S., Gohar, M., Slamti, L., Nel, P. and Lereclus, D. (2003). The *Bacillus thuringiensis* PlcR-regulated gene *inhA2* is necessary, but not sufficient, for virulence. *J. Bacteriol*. **185,** 2820–2825.

Gassler, C.S., Ryan, M., Liu, T., Griffith, O.H. and Heinz, D.W. (1997). Probing the roles of active site residues in phosphatidylinositol-specific phospholipase C from *Bacillus cereus* by site-directed mutagenesis. *Biochemistry* **36,** 12802–12813.

Gaur, A.H., Patrick, C.C., McCullers, J.A., Flynn, P.M., Pearson, T.A., Razzouk, B.I., Thompson, S.J. and Shenep, J.L. (2001). *Bacillus cereus* bacteremia and meningitis in immunocompromised children. *Clin. Infect. Dis*. **32,** 1456–1462.

Ghelardi, E., Celandroni, F., Salvetti, S., Beecher, D.J., Gominet, M., Lereclus, D., Wong, A.C. and Senesi, S. (2002). Requirement of *flhA* for swarming differentiation, flagellin export, and secretion of virulence-associated proteins in *Bacillus thuringiensis. J. Bacteriol*. **184,** 6424–6433.

Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wachter, M., Kreft, J. and Goebel, W. (1989). A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. *J. Bacteriol*. **171,** 744–753.

Gohar, M., Okstad, O.A., Gilois, N., Sanchis, V., Kolstø, A.B. and Lereclus, D. (2002). Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. *Proteomics*. **2,** 784–791.

Gominet, M., Slamti, L., Gilois, N., Rose, M. and Lereclus, D. (2001). Oligopeptide permease is required for expression of the *Bacillus thuringiensis plcR* regulon and for virulence. *Mol. Microbiol*. **40,** 963–975.

Granum, P.E. (1994). *Bacillus cereus* and its toxins. *Soc. Appl. Bacteriol. Symp. Ser*. **23,** 61S–66S.

Granum, P.E. (1997). *Bacillus cereus*. In: *Food Microbiology Fundamentals and Frontiers*, (eds. M.P. Doyle, L.R. Beuchat, and T.J. Montville), pp. 327–336 ASM, Washington.

Granum, P.E. (2001). *Bacillus cereus*. In: *Food Microbiology Fundamentals and Frontiers*, (eds. M.P. Doyle, L.R. Beuchat, and T.J. Montville), pp. 373–381 ASM, Washington.

Granum, P.E., Brynestad, S., O'Sullivan, K. and Nissen, H. (1993). The enterotoxin from *Bacillus cereus*: production and biochemical characterization. *Neth. Milk and Dairy J*. **47,** 63–70.

Granum, P.E. and Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Lett*. **157,** 223–228.

Granum, P.E., O'Sullivan, K. and Lund, T. (1999). The sequence of the non-hemolytic enterotoxin operon from *Bacillus cereus. FEMS Microbiol. Lett*. **177,** 225–229.

Gray, J., George, R.H., Durbin, G.M., Ewer, A.K., Hocking, M.D. and Morgan, M.E. (1999). An outbreak of *Bacillus cereus* respiratory tract infections on a neonatal unit due to contaminated ventilator circuits. *J. Hosp. Infect*. **41,** 19–22.

Guinebretière, M.H., Broussolle, V. and Nguyen-The, C. (2002). Enterotoxigenic profiles of food poisoning and food-borne *Bacillus cereus* strains. *J. Clin. Microbiol*. **40,** 3053–3056.

Guinebretière, M.H. and Sanchis, V. (2003). *Bacillus cereus sensu lato. Bull. Soc. Fr. Microbiol*. **18,** 95–103.

Hansen, B.M., Hoiby, P.E., Jensen, G.B. and Hendriksen, N.B. (2003). The *Bacillus cereus* bceT enterotoxin sequence reappraised. *FEMS Microbiol. Lett*. **223,** 21–24.

Hardy, S.P., Lund, T. and Granum, P.E. (2001). CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia. *FEMS Microbiol. Lett*. **197,** 47–51.

Heinrichs, J.H., Beecher, D.J., Macmillan, J.D. and Zilinskas, B.A. (1993). Molecular cloning and characterization of the *hblA* gene encoding the B component of hemolysin BL from *Bacillus cereus. J. Bacteriol*. **175,** 6760–6766.

Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I. and Kolstø, A.B. (2000). *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. *Appl. Environ. Microbiol*. **66,** 2627–2630.

Helgason, E., Tourasse, N.J., Meisal, R., Caugant, D.A. and Kolstø A.B. (2004). Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. *Appl. Environ. Microbiol*. **70,** 191–201.

Hondal, R.J., Zhao, Z., Kravchuk, A.V., Liao, H., Riddle, S.R., Yue, X., Bruzik, K.S. and Tsai, M.D. (1998). Mechanism of phosphatidylinositol-specific phospholipase C: a unified view of the mechanism of catalysis. *Biochemistry* **37,** 4568–4580.

Horwood, P.F., Burgess, G.W. and Oakey, H.J. (2004). Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. *FEMS Microbiol. Lett*. **236,** 319–324.

Hsueh, P.R., Teng, L.J., Yang, P.C., Pan, H.L., Ho, S.W. and Luh, K.T. (1999). Nosocomial pseudoepidemic caused by *Bacillus cereus* traced to contaminated ethyl alcohol from a liquor factory. *J. Clin. Microbiol*. **37,** 2280–2284.

Hu, X., Hansen, B.M., Eilenberg, J., Hendriksen, N.B., Smidt, L., Yuan, Z. and Jensen, G.B. (2004). Conjugative transfer, stability, and expression of a plasmid encoding a *cry1Ac* gene in *Bacillus cereus* group strains. *FEMS Microbiol. Lett*. **231,** 45–52.

Jaaskelainen, E.L., Teplova, V., Andersson, M.A., Andersson, L.C., Tammela, P., Andersson, M.C., Pirhonen, T.I., Saris, N.E., Vuorela, P. and Salkinoja-Salonen, M.S. (2003). *In vitro* assay for human toxicity of cereulide, the emetic mitochondrial toxin produced by food poisoning *Bacillus cereus*. *Toxicol. In Vitro* **17,** 737–744.

Jensen, G.B., Hansen, B.M., Eilenberg, J. and Mahillon, J. (2003). The hidden lifestyles of *Bacillus cereus* and relatives. *Environ. Microbiol*. **5,** 631–640.

Johansen, T., Bjorkoy, G., Overvatn, A., Diaz-Meco, M.T., Traavik, T. and Moscat, J. (1994). NIH 3T3 cells stably transfected with the gene encoding phosphatidylcholine-hydrolyzing phospholipase C from *Bacillus cereus* acquire a transformed phenotype. *Mol. Cell Biol*. **14,** 646–654.

Klichko, V.I., Miller, J., Wu, A., Popov, S.G. and Alibek, K. (2003). Anaerobic induction of *Bacillus anthracis* hemolytic activity. *Biochem. Biophys. Res. Commun*. **303,** 855–862.

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect*. **2,** 189–198.

Kramer, J. and Gibert, R. (1989). *Bacillus cereus* and other *Bacillus* species. In: *Foodborne Pathogenic Bacteria* (ed. M. Doyle), pp. 21–70. Marcel Dekker, New York.

Kravchuk, A.V., Zhao, L., Bruzik, K.S. and Tsai, M.D. (2003). Engineering a catalytic metal-binding site into a calcium-independent, phosphatidylinositol-specific phospholipase C leads to enhanced stereoselectivity. *Biochemistry* **42,** 2422–2430.

Kuppe, A., Evans, L.M., McMillen, D.A. and Griffith, O.H. (1989). Phosphatidylinositol-specific phospholipase C of *Bacillus cereus*: cloning, sequencing, and relationship to other phospholipases. *J. Bacteriol*. **171,** 6077–6083.

Lechner, S., Mayr, R., Francis, K.P., Pruss, B.M., Kaplan, T., Wiessner-Gunkel, E., Stewart, G.S. and Scherer, S. (1998). *Bacillus weihenstephanensis sp. nov*. is a new psychrotolerant species of the *Bacillus cereus* group. *Int. J. Syst. Bacteriol*. **48,** 1373–1382.

Lehto, M.T. and Sharom, F.J. (2002). PI-specific phospholipase C cleavage of a reconstituted, GPI-anchored protein: modulation by the lipid bilayer. *Biochemistry* **41,** 1398–1408.

Leppla, S. (1999). The bifactorial *Bacillus anthracis* lethal and oedema toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer) pp. 243–263. Academic Press, London.

Lereclus, D., Agaisse, H., Gominet, M., Salamitou, S. and Sanchis, V. (1996). Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. *J. Bacteriol*. **178,** 2749–2756.

Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S. and Gominet, M. (2000). Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. *Int. J. Med. Microbiol*. **290,** 295–299.

Lindbäck, T., Fagerlund, A., Rødland, M. S. and Granum, P. E. (2004). Characterization of the *Bacillus cereus* Nhe enterotoxin. *Microbiology* **150,** 3959–3967.

Lund, T., De Buyser, M.L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. **38,** 254–261.

Lund, T. and Granum, P.E. (1996). Characterization of a non-hemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiol. Lett*. **141,** 151–156.

Lund, T. and Granum, P.E. (1997). Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. *Microbiology* **143,** 3329–3336.

Margot, P., Wahlen, M., Gholamhoseinian, A., Piggot, P., Karamata, D. and Gholamhuseinian, A. (1998). The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. *J. Bacteriol*. **180,** 749–752.

Margulis, L., Jorgensen, J.Z., Dolan, S., Kolchinsky, R., Rainey, F.A. and Lo, S.C. (1998). The *Arthromitus* stage of *Bacillus cereus*: intestinal symbionts of animals. *Proc. Natl. Acad. Sci. USA* **95,** 1236–1241.

Martin, S.F., Follows, B.C., Hergenrother, P.J. and Trotter, B.K. (2000). The choline binding site of phospholipase C (*Bacillus cereus*): insights into substrate specificity. *Biochemistry* **39,** 3410–3415.

Menestrina, G., Serra, M.D. and Prevost, G. (2001). Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* **39,** 1661–1672.

Mignot, T., Mock, M., Robichon, D., Landier, A., Lereclus, D. and Fouet, A. (2001). The incompatibility between the PlcR- and AtxA-controlled regulons may have selected a nonsense mutation in *Bacillus anthracis*. *Mol. Microbiol*. **42,** 1189–1198.

Miles, G., Bayley, H. and Cheley, S. (2002). Properties of *Bacillus cereus* hemolysin II: a heptameric transmembrane pore. *Protein Sci*. **11,** 1813–1824.

Miller, J.M., Hair, J.G., Hebert, M., Hebert, L., Roberts, F.J., Jr. and Weyant, R.S. (1997). Fulminating bacteremia and pneumonia due to *Bacillus cereus*. *J. Clin. Microbiol*. **35,** 504–507.

Nakamura, L.K. and Jackson, M.A. (1995). Clarification of the taxonomy of *B. mycoides*. *Int. J. System. Bacteriol*. **45,** 46–49.

Nakamura, L.K. (1998). *Bacillus pseudomycoides* sp. nov. *Int. J. Syst. Bacteriol*. **48,** 1031–1035.

Obama, T., Fujii, S., Ikezawa, H., Ikeda, K., Imagawa, M. and Tsukamoto, K. (2003a). His151 and His296 are the acid-base catalytic residues of *Bacillus cereus* sphingomyelinase in sphingomyelin hydrolysis. *Biol. Pharm. Bull*. **26,** 920–926.

Obama, T., Kan, Y., Ikezawa, H., Imagawa, M. and Tsukamoto, K. (2003b). Glu-53 of *Bacillus cereus* sphingomyelinase acts as an indispensable ligand of Mg2+ essential for catalytic activity. *J. Biochem*. (Tokyo) **133,** 279–286.

Palmer, M. (2001). The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon* **39,** 1681–1689.

Pomerantsev, A.P., Kalnin, K.V., Osorio, M. and Leppla, S.H. (2003). Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. *Infect. Immun*. **71,** 6591–6606.

Pomerantsev, A.P., Pomerantseva, O.M. and Leppla, S.H. (2004). A spontaneous translational fusion of *Bacillus cereus* PlcR and PapR activates transcription of PlcR-dependent genes in *Bacillus anthracis* via binding with a specific palindromic sequence. *Infect. Immun*. **72,** 5814–5823.

Prevost, G. (1999). The bi-component staphylococcal leukocidins and γ-hemolysins (toxins). In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.H. Freer and J.E. Alouf), pp. 402–418. Academic Press, London.

Ryan, P.A., Macmillan, J.D. and Zilinskas, B.A. (1997). Molecular cloning and characterization of the genes encoding the L1 and L2 components of hemolysin BL from *Bacillus cereus*. *J. Bacteriol*. **179,** 2551–2556.

Salamitou, S., Ramisse, F., Brehelin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. and Lereclus, D. (2000). The *plcR*

regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology* **146,** 2825–2832.

Schlax, P.J. and Worhunsky, D.J. (2003). Translational repression mechanisms in prokaryotes. *Mol. Microbiol*. **48,** 1157–1169.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev*. **62,** 775–806.

Schoeni, J.L. and Wong, A.C. (1999). Heterogeneity observed in the components of hemolysin BL, an enterotoxin produced by *Bacillus cereus*. *Int. J. Food Microbiol*. **53,** 159–167.

Shannon, J.G., Ross, C.L., Koehler, T.M. and Rest, R.F. (2003). Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin. *Infect. Immun*. **71,** 3183–3189.

Shany, S., Bernheimer, A.W., Grushoff, P.S. and Kim, K.S. (1974). Evidence for membrane cholesterol as the common binding site for cereolysin, streptolysin O, and saponin. *Mol. Cell. Biochem*. **3,** 179–186.

Sinev, M.A., Budarina, Z.I., Gavrilenko, I.V., Tomashevskii, A.I. and Kuz'min, N.P. (1993). Evidence of the existence of hemolysin II from *Bacillus cereus*: cloning the genetic determinant of hemolysin II. *Mol.Biol*. (Mosk.) **27,** 1218–1229.

Slamti, L. and Lereclus, D. (2002). A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the *Bacillus cereus* group. *EMBO J*. **21,** 4550–4559.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Steinthorsdottir, V., Halldorsson, H. and Andresson, O.S. (2000). *Clostridium perfringens* beta-toxin forms multimeric transmembrane pores in human endothelial cells. *Microb. Pathog*. **28,** 45–50.

Stenfors, L.P. and Granum, P.E. (2001). Psychrotolerant species from the *Bacillus cereus* group are not necessarily *Bacillus weihenstephanensis*. *FEMS Microbiol. Lett*. **197,** 223–228.

Stenfors, L.P., Mayr, R., Scherer, S. and Granum, P.E. (2002). Pathogenic potential of fifty *Bacillus weihenstephanensis* strains. *FEMS Microbiol. Lett*. **215,** 47–51.

Thomas, D.J., Morgan, J.A., Whipps, J.M. and Saunders, J.R. (2001). Plasmid transfer between *Bacillus thuringiensis subsp. israelensis* strains in laboratory culture, river water, and dipteran larvae. *Appl. Environ. Microbiol*. **67,** 330–338.

Turnbull, P.C. (1999). Definitive identification of *Bacillus anthracis*—a review. *J. Appl. Microbiol*. **87,** 237–240.

Wehbi, H., Feng, J. and Roberts, M.F. (2003). Water-miscible organic cosolvents enhance phosphatidylinositol-specific phospholipase C phosphotransferase as well as phosphodiesterase activity. *Biochim. Biophys. Acta* **1613,** 15–27.

Yamada, A., Tsukagoshi, N., Udaka, S., Sasaki, T., Makino, S., Nakamura, S., Little, C., Tomita, M. and Ikezawa, H. (1988). Nucleotide sequence and expression in *Escherichia coli* of the gene coding for sphingomyelinase of *Bacillus cereus*. *Eur. J. Biochem*. **175,** 213–220.

Yokomatsu, T., Murano, T., Akiyama, T., Koizumi, J., Shibuya, S., Tsuji, Y., Soeda, S. and Shimeno, H. (2003). Synthesis of non-competitive inhibitors of sphingomyelinases with significant activity. *Bioorg. Med. Chem. Lett*. **13,** 229–236.

Zhang, X., Wehbi, H. and Roberts, M.F. (2004). Cross-linking phosphatidylinositol-specific phospholipase C traps two activating phosphatidylcholine molecules on the enzyme. *J. Biol. Chem*. **279,** 20490–20500.

Zhao, L., Liao, H. and Tsai, M.D. (2004). The catalytic role of aspartate in a short strong hydrogen bond of the Asp274-His32 catalytic dyad in phosphatidylinositol-specific phospholipase C can be substituted by a chloride ion. *J. Biol. Chem*. **279,** 31995–32000.

CHAPTER

# 47

# Uropathogenic *Escherichia coli* cytolysins

*Tobias A. Oelschlaeger and Jörg Hacker*

## INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) strains encode a variety of toxins. One toxin type is the cytolethal distending toxin. It is encoded by three genes (*cdtABC*) and blocks the cell cycle in G2 phase (Scott and Kaper, 1994). The block is due to keeping the mitosis promoting factor in an inactive form (Comayras *et al*., 1997). Another toxin type is CNF (cytotoxic necrotizing factor), for which two variants are known. CNF1 is chromosomally encoded, and CNF2 is plasmid encoded (Oswald *et al*., 1989). This toxin induces multinucleation in cell culture and causes necrosis in rabbit skin (De Rycke *et al*., 1990). Furthermore, CNF1 was shown to be an important virulence factor of UPEC and to activate Rho GTPases (Rippere-Lampe *et al*., 2001).

Another group of toxic proteins secreted by UPEC are members of the SPATE (serine protease autotransporters of *Enterobacteriaceae*) family (Dutta *et al*., 2002). Two SPATE types have been identified so far in UPEC. One is Sat (secreted autotransporter toxin), and the other is PicU (Parham *et al*., 2004; Guyer *et al*., 2000). All SPATE are characterized by the presence of a serine protease active site motif in the passenger domain, which is necessary for phenotypic functions. For Sat, vacuolation of target cells was demonstrated *in vitro* and *in vivo* (Guyer *et al*., 2002). However, most frequently UPEC produce a cytolysin toxin. Genetic analysis revealed that UPEC often contain the cytolysin A gene (*clyA*, *hlyE*, *sheE*). However, *clyA* of UPEC is non-functional in all UPEC strains tested, due to deletion(s) in the *clyA* locus. In contrast, a functional allele of *clyA* is present in many strains of intestinal *E. coli* pathotypes and even in *E. coli* K-12 strains (Ludwig *et al*., 2004). Therefore, the cytolytic activity of UPEC is not due to ClyA but to the expression of α-hemolysin.

This cytolysin is termed α-hemolysin because its cytolytic activity can be demonstrated easily by hemolysis. It is the prototype of the RTX (repeats-in-toxin) family of exoproteins produced by a diverse group of Gram-negative pathogens. Other members of this toxin family are the hemolysin of enterohemorrhagic *E. coli* (EhxA) (Schmidt *et al*., 1996), the leukotoxin of *Pasteurella haemolytica* (Strathdee and Lo, 1987), and the hemolysins and leukotoxins of *Actinobacillus* spp.(Welch *et al*., 1995). The toxins of this family are grouped on the basis of their specificity: Hemolysins exhibit little target cell specificity, while leukotoxins have pronounced species- or cell-specific effects. All of these toxins share up to 70% sequence identity with *E. coli* HlyA. Other characteristics shared by this toxin family are (i) post-translational modification, (ii) a C-terminal calcium-binding domain of acidic glycin-rich repeats, which has the consensus sequence GGXGXDX[L/I/V/W/Y/F] (X stands for any amino acid) and that has led to the RTX family nomenclature, and (iii) secretion by type I secretion systems (Koronakis and Hughes, 1996; Lilie *et al*., 2000). Posttranslational modification and calcium binding are absolute requirements for cytotoxic activity (Rowe *et al*., 1994; Ostolaza *et al*., 1995) of this toxin.

## GENETICS OF α-HEMOLYSIN

The generic RTX toxin operon consists of four genes. The genes required for hemolysis by UPEC are *hlyC*, *hlyA*, *hlyB*, and *hlyD* in transcriptional order (Hacker and Hughes, 1985). HlyC is responsible for the post-translational modification of HlyA by internal acylation of two lysine residue epsilon-amino groups. Hemolysin produced in the absence of HlyC is biologically inactive. HlyB and D are essential for HlyA secretion (Wagner *et al*., 1983). HlyB, an inner membrane traffic ATPase containing an ATP-binding cassette, is responsible for supplying the energy for the HlyA secretion and for the translocation across the inner membrane (Koronakis *et al*., 1995). The transport through the periplasm of HlyA is mediated by HlyD, a member of the membrane fusion proteins family. HlyD is anchored in the inner membrane and forms a channel connecting HlyB in the inner membrane and TolC in the outer membrane (Schlor *et al*., 1997). TolC is a general outer membrane protein that is part of at least four different export systems (Paulsen *et al*., 1997; Zgurskaya and Nikaido, 2000). The *hly*-determinant of UPEC is either encoded by transmissible plasmids or by chromosomally located pathogenicity islands, suggesting that this determinant can be transmitted by horizontal gene transfer among Gram-negative bacteria.

A well-studied example of an α-hemolysin encoding plasmid is pHly152 (Noegel *et al*., 1981). Furthermore, the determinant can be lost due to deletion of the whole pathogenicity island or plasmid, resulting in severely attenuated mutants (Knapp *et al*., 1986). Certain UPEC strains contain even two α-hemolysin determinants on two different pathogenicity islands (Nagy *et al*., 2000). This is the case for UPEC strain 536 (Blum *et al*., 1994), as well as for strain J96 (Blum *et al*., 1995; Swenson *et al*. 1996). Also for the third prototypic UPEC strain, CFT073, the α-hemolysin determinant is encoded by a pathogenicity island (Guyer *et al*., 1998). Typically, the pathogenicity islands are inserted in tRNA-genes. The α-hemolysin genes encoding pathogenicity islands of UPEC strain 536 are located in *selC* and *leuX*, of UPEC strain J96 in *pheV* and *pheR*, and in UPEC strain CFT073 in *metV* (Kao *et al*., 1997; Swenson *et al*., 1996; Blum *et al*., 1994). As a remnant of the probable phage origin, these pathogenicity islands each harbor also an integrase gene (Middendorf *et al*., 2004). The integrase is made responsible not only for the integration of the pathogenicity island after horizontal transfer, but also for deletion of the pathogenicity island by recognizing the direct repeats flanking this genetic unit.

## REGULATION OF α-HEMOLYSIN EXPRESSION

The expression of the α-hemolysin in *E. coli* is regulated by environmental signals. Most expression studies made use of plasmids pHly152 or pANN202-312 (Vogel *et al*., 1988; Welch and Pellet, 1988). The *hlyCABD* operon is transcribed from a promoter located upstream of *hlyC*, and several mechanisms are in place for its regulation. The transcription is strongly polar, owing to the presence of a rho-independent terminator in the *hlyA-hlyB* intergenic region (Welch and Pallett, 1988). This termination is suppressed by the elongation protein RfaH and a cis-acting 5′-DNA sequence termed the JUMPstart element or ops (operon-polarity suppressor) element, which must act together to allow the efficient transcription of the entire *hly* operon (Bailey *et al*., 1992). One important signal for regulation of *hly*-operon expression is temperature. Low temperature represses expression (Mourino *et al*., 1996). Another signal influencing *hly*-expression is osmolarity. The expression of the hemolysin genes is repressed when *E. coli* cells grow in a medium-to-high osmolarity medium (Carmona *et al*., 1993). Mediators of *hly* expression are H-NS and Hha. H-NS is a major component of the bacterial nucleoid and plays a central role as a modulator of gene expression in response to osmolarity and temperature changes (Atlung and Ingmer, 1997). It binds preferentially to curved DNA and is able to generate bends in non-curved DNA, thereby affecting the expression of a large number of genes, and it is considered a general negative transcriptional regulator (Hommais *et al*., 2001). H-NS binds *in vitro* more efficiently to sequences upstream of *hlyC* at low temperature, correlating with the more efficient transcriptional repression caused by H-NS at low temperature and with the hemolytic phenotype of *hns* mutants (Madrid *et al*., 2002).

The two binding sites for H-NS are located upstream of *hlyC*. One of them, more than 1.5 kb upstream of *hlyC*, is within the regulatory sequence termed *hlyR*, which contains an antiterminator element (the *ops* element). Deletion of *hlyR* results in strong repression of hemolysin expression (Vogel *et al*., 1988; Nieto *et al*., 1996). The other H-NS binding site is close to *hlyC*, partially overlapping the promoter region of the *hly* operon. The role of the *IS*2 element separating both H-NS binding sites remains unclear, since no *hly* promoter function has been assigned to this element and its deletion has only a moderate effect on *hly* transcription, which is most likely due to the resulting topological modifications (Madrid *et al*., 2002). The Hha protein belongs to a family of modulators, which include the

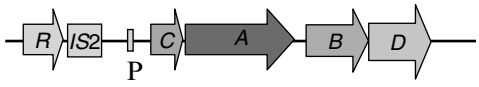



**FIGURE 47.1** Physical map of the α-hemolysin determinant. Arrows indicate open reading frames/genes for the regulatory sequence (*R*), the activator gene (*C*) responsible for internal acylation of the α-hemolysin encoded by *hlyA* (*A*), the gene for the inner membrane ATPase (*B*) and the gene for the membrane fusion protein (*D*). The orientation of the arrows reflects the direction of transcription. *IS*2: Insertion sequence 2; P: promoter.

YmoA protein of *Yersinia enterocolitica*, regulating expression of different virulence factors in a temperature-dependent manner (Cornelis *et al*., 1991). Hha interacts with H-NS, and this complex is an important component in the thermo-osmotic regulation of the *hly* operon. This view is supported by the observation of loss of both thermo- and osmoregulation in *hha/hns* double mutants (Nieto *et al*., 2000). Expression of Hha itself is modulated by osmolarity in that its expression reaches a maximum in the exponential growth phase in LB medium, but depletion of NaCl leads to a significant decrease in expression (Mourino *et al*., 1998). The Hha/H-NS complex bound to both H-NS binding sites could occlude the *ops* sequence and the promoter region as well, thus repressing transcription and abolishing the antitermination effect of *ops* (Madrid *et al*., 2002). However, the Hha- and H-NS-mediated repression of the *hly* operon is not an on-off process but rather a gradual one. This conclusion is drawn from the observation of an increase in α-hemolysin production by hns mutants grown under 37°C compared to isogenic wild-type strains grown under identical conditions (Nieto *et al*., 2000). Obviously, the H-NS- and Hha-regulated repression of α-hemolysin synthesis is influenced by different environmental factors that cause variations in the physicochemical parameters of DNA or sequestering or degradation of H-NS.

## ACTIVATION OF α-HEMOLYSIN

The HlyA toxin is synthesized as an inactive 1,024-residue protoxin, pro-HlyA, which is activated intracellularly in the HlyA-producing bacterial cell to the mature toxin by the action of the co-synthesized HlyC. HlyC is a homodimeric acyltransferase using acyl-acyl carrier protein (acyl-ACP) as the fatty acid donor. Indeed, two internal fatty-acylated lysine residues, K564 and K690, were identified, and resistance of the acylation to hydroxylamine suggested that the fatty acid is amide linked. Acylation of both sites is required for *in vivo* toxin hemolytic activity (Stanley *et al*., 1994; Stanley *et al*., 1996). Also, the other HlyA-related toxins secreted by pathogenic Gram-negative bacteria all require HlyC-type protoxin activation and many (PvxA, MmxA, ApxIA, and LktA) have been activated by *E. coli* HlyC (Forestier and Welch, 1990; Gygi *et al*., 1990; Koronakis *et al*., 1987). The ability of HlyC to transfer an acyl group to an internal lysine residue or a target protein distinguishes HlyC from all other bacterial acyltransferases. This is strongly supported by the fact that *hlyA*$^+$*hlyC* strains are non-hemolytic, despite the various constitutive acyltransferases present in *E. coli*, which are obviously unable to substitute for HlyC to even a small degree. This uniqueness is also reflected in the lack of significant sequence homology of HlyC with known acyltransferases, such as the lipid A acyltransferases (Clementz *et al*., 1997). Myristoyl-ACP gave the highest hemolytic activity of HlyA acylated *in vitro* with a range of fatty acids and *in vivo* HlyA is indeed predominantly myristoylated (Issartel *et al*., 1991; Ludwig *et al*., 1996). Two independent HlyC recognition domains have been identified on pro-HlyA, each of which spans one of the target lysine residues (Stanley *et al*. 1996). Although both HlyC recognition domains of HlyA have the same function, there is surprisingly little primary sequence identity (only 21%). The lack of identity between both recognition domains in pro-HlyA and corresponding sites on related protoxins currently deters an explanation for basis of HlyA recognition by HlyC. It is worth mentioning that both membrane immunoglobulin and insulin receptors appear to possess identical amide-linked, internal fatty acylation, as found on HlyA. In addition, the Ras and Src componentsof these signaling pathways, and the heterotrimeric G-proteins and their coupled receptors, all have acylated residues (Stanley *et al*., 1998). It is therefore speculated that cytokine-inducing proteins produced by bacteria as HlyA are the evolutionary precursors of mammalian cytokines (Henderson *et al*., 1996).

## THE α-HEMOLYSIN SECRETION SYSTEM

Pro-HlyA is synthesized and activated in the cytoplasm of the bacterial cell and subsequently secreted across both membranes. The secretion system of α-hemolysin is the prototype of type I secretion systems. Type I secretion systems are characterised by (i) containing only three different transport components, two in the inner membrane, which are specific for the passenger protein (here HlyA), and one that forms a general pore in the outer membrane; (ii) these proteins

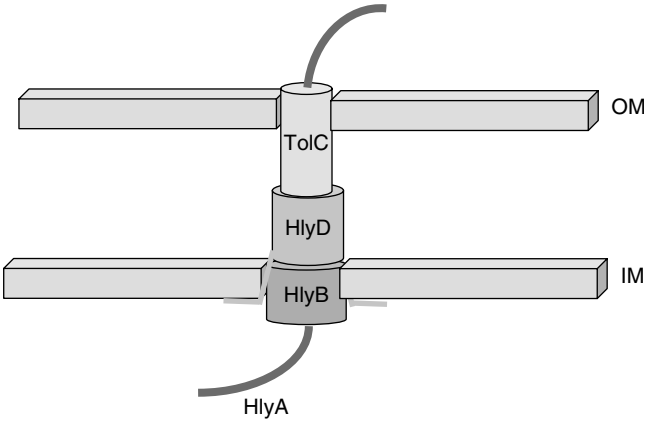



**FIGURE 47.2** Model of the α-hemolysin export apparatus. After interaction of HlyA with HlyB and HlyD, the inner membrane ATPase HlyB, the membrane fusion protein HlyD, and the outer membrane protein TolC connect and form a channel-tunnel for the secretion of HlyA. OM: outer membrane; IM: inner membrane.

form a tunnel that links the inner and outer membrane; (iii) the passenger proteins are secreted directly into the extracellular medium without the formation of periplasmic intermediates; (iv) the secretion signal recognized by these secretion systems are located at the C-terminus of the secreted protein; (v) in general, the secretion signal is not cleaved off during or after secretion (for a recent review see Andersen 2003).

The activation by acylation is not necessary for secretion of HlyA, because pro-HlyA is secreted as efficiently as mature HlyA (Ludwig *et al*., 1987). The HlyA secretory apparatus comprises HlyB (an inner membrane traffic ATPase), HlyD (an inner membrane protein that bridges the periplasm by connecting the inner with the outer membrane), and TolC (an outer membrane protein). In *E. coli*, as in most other pathogens, TolC is encoded by a gene separated from the *hlyCABD* operon (Gentschev *et al*., 2002).

The first component, HlyB, of the hemolysin type I secretion system belongs to the ATP-binding cassette (ABC) superfamily of eukaryotic and prokaryotic protein transporters (Holland and Blight, 1999). This inner membrane protein couples ATP hydrolysis to the export of the substrate. Experimental data obtained suggest that HlyB is inserted in the inner membrane by eight hydrophobic α-helical transmembrane domains. These transmembrane domains extend from amino acid positions 39 to 432 of HlyB (Koronakis *et al*., 1995; Gentschev and Goebel, 1992).

The second component (HlyD) is one of the best-characterized members of the membrane fusion protein (MFP) family. HlyD is anchored in the cytoplasmic membrane by a single transmembrane domain and possesses a large periplasmic domain within the carboxy-terminal 100 amino acids, which are highly conserved among MFPs (Schülein *et al*., 1992). A recent study grouped the members of the MFP family within the superfamily of periplasmic efflux proteins (PEP) (Johnson and Church, 1999).

The third component (TolC) is a general outer membrane protein (OMP) that is part of at least four different export systems (Zgurskaya and Nikaido, 2000). The crystal structure of TolC shows that its trimeric state forms a trans-periplasmic channel-tunnel with an internal diameter of 35 Å and is about 140 Å in length, comprising a 40 Å-long OM β-barrel (the channel domain) anchoring a contiguous 100 Å-long α-helical barrel that projects across the periplasmic space (the tunnel domain) (Koronakis *et al*., 2000; Andersen *et al*., 2001).

HlyA carries a C-terminal secretion signal located with the last 50–60 amino acids. The precise nature of the secretion signal remains basically unknown. However, a peptide consisting of just the C-terminal 60 amino acids of HlyA is secreted in the presence of HlyB-HlyD-TolC with the same efficiency as HlyA itself. In contrast, in some other type I–secreted proteins, the repeats within these proteins are required for efficient secretion (Jarchau *et al*., 1994).

## RECEPTOR(S) AND MECHANISM(S) OF ACTION

The term hemolysin for α-hemolysin of *E. coli* was coined because of the ability of mature HlyA to efficiently lyse erythrocytes; even so, α-hemolysin is able to lyse a great variety of cell types from various hosts. It is also suggested that leukocytes are the main target for α-hemolysin. This finding made it hard to imagine a receptor for α-hemolysin (e.g., on erythrocytes), despite the general rule for bacterial toxins to possess domains for target cell recognition. However, for the HlyA-sensitive HL-60 cell line, two polypeptides were identified (CD11a and CD18) to which HlyA binds, and a monoclonal antibody specific for these polypeptides protected HL-60 cells from the cytolysis by HlyA. CD11a and CD18 are subunits of lymphocyte-function-associated antigen 1 (LFA-1), a $\beta_2$ integrin that is found in circulating leukocytes, but not on cells of non-hematopoetic origin. On erythrocytes, glycophorin, present in high numbers in the cytoplasmic membrane of this cell type, has been identified as the receptor for HlyA (Cortajarena *et al*., 2001). Furthermore, the corresponding receptor-binding domain in α-hemolysin was identified as the region comprising residues 914 to 936 in HlyA. A deletion mutant of HlyA lacking these residues showed a hemolytic activity 10,000-fold decreased compared to the wild-type (Cortajarena *et al*., 2003).

Lysis of target cells, e.g., erythrocytes, occurs via formation of transmembrane pores in the cytoplasmic membrane (Bhakdi *et al*. 1986). The pores seemed to be formed by oligomeres of HlyA (Benz *et al*., 1989). In addition, the identification of several different functional regions responsible for pore formation, calcium-dependent membrane binding, and pore stability were reported. The C-terminal half of HlyA contains 12 repeats of the consensus nonapeptide X-Leu-X-Gly-Gly-X-X-Gly-Asp-Asp-Asp. The repeat sequence (residues 739–849) represents the $Ca^{2+}$-binding domain and is essential for function. Eight transmembrane segments of the hydrophobic region of HlyA were predicted to be part of the pore-forming structure (Ludwig *et al*., 1988; Ludwig *et al*., 1991). The HlyA pores were reported to be cation-specific with an inner diameter of 1–2 nm (Benz *et al*., 1989). Pore formation was further supported by direct visualization in model systems and by path clamp characterization with human macrophages (Menestrina *et al*., 1996).

This view of α-hemolysin action was challenged by Soloaga *et al*. (1999). They presented data supporting a model for the membrane-inserted HlyA to occupy only the outer layer of phospholipids of the host cell cytoplasmic membrane and not to form a transmembrane pore. The insertion into the outer layer of the membrane is mediated by 10 amphipathic helices of HlyA. This interaction would also lead to cell leakage or lysis of host cells by inducing an increase in the lateral pressure in the outer monolayer of the membrane lipids. Beyond a certain increase, the monolayer will reach a point of transient breakdown and subsequent leakage of contents (Soloaga *et al*., 1999).

Whatever model for HlyA interaction with host cell membranes comes closest to reality, the effects of the interaction is more than just cell lysis. The release of inflammatory mediators from human polymorphonuclear granulocytes as $O_2^-$ and β-glucuronidase, as well as leukotriene generation, was reported by König *et al*. (1999). In addition, these authors demonstrated histamine release from human lymphocyte/monocyte basophil cells and serotonin release and 12-hydroxyeicosatetronic acid generation from human platelets. All these effects were dependent on wild-type α-hemolysin and not observed with HlyA-mutants deficient in pore-forming activity (König *et al*., 1994). In addition, HlyA induced massive shedding of receptors for interleukin 6 (IL-6R) and lipopolysaccharide (CD14). Shedding was not PKC-dependent (Walev *et al*., 1996). Trans-signaling by soluble IL-6R and soluble CD14 is known to dramatically broaden the spectrum of host cells for IL-6 and lipopolysaccharide and is thus an important mechanism underlying their systemic inflammatory effects. Hence, these findings highlight a mechanism by which HlyA can promote inflammatory processes. More recently, HlyA was demonstrated to induce low-frequency oscillatory $[Ca^{2+}]_i$ response in target primary rat renal epithelial cells. The observed response depended on calcium influx through L-type calcium channels, as well as from internal stores gated by inositol triphosphate. Internal calcium oscillations induced in a renal epithelial cell line stimulated production of cytokines interleukin 6 and interleukin 8. Obviously, α-hemolysin can act as an inducer of an oscillating second messenger response in target cells, e.g., kidney cells, and thereby fine-tune gene expression during the inflammatory response (Uhlen *et al*., 2000).

## CONCLUSION

The α-hemolysin of UPEC seems to be important for urinary tract infections by UPEC. However, the *hly*-determinant is also found in a considerable number of fecal *E. coli* isolates, which express the α-hemolysin, at least *in vitro*. It has been intensively studied by several research groups for the past few decades, but many questions regarding its activation, secretion, and mode of action are still open. Why is acylation of HlyA essential for its hemolytic action? What is the nature of the C-terminal secretion signal? Does HlyA really form pores in host cell membranes? How can HlyA have so many different effects on host cells? More effort will be needed to answer these and other questions regarding HlyA. These answers will not only further our academic knowledge, but will form the basis for more efficient treatment and hopefully the prevention of urinary tract infections.

## REFERENCES

Andersen, C., Hughes, C. and Koronakis, V. (2001). Protein export and drug efflux through bacterial channel-tunnels. *Curr. Opin. Cell Biol*. **13,** 412–416.

Andersen, C. (2003). Channel-tunnels: outer membrane components of type I secretion systems and multidrug efflux pumps of Gram-negative bacteria. *Rev. Physiol. Biochem. Pharmacol*. **147,** 122–165.

Atlung, T. and Ingmer, A. (1997). H-NS: a modulator of environmental regulated gene expression. *Mol. Microbiol*. **24,** 7–17.

Bailey, M.J., Koronakis, V., Schmoll, T. and Hughes, C. (1992). *Escherichia coli* HlyT protein, a transcriptional activator of hemolysin synthesis and secretion, is encoded by the *rfaH* (*sfrB*) locus required for expression of sex factor and lipopolysaccharide genes. *Mol. Microbiol*. **6,** 1003–1012.

Benz, R., Schmid, A., Wagner, W. and Goebel, W. (1989). Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. *Infect. Immun*. **57,** 887–895.

Blum, G., Ott, M., Lischewski, A., Ritter, A., Imrich, H., Tschäppe, H. and Hacker, J. (1994). Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infect. Immun*. **62,** 606–614.

Blum, G., Falbo, V., Caprioli, A. and Hacker, J. (1995). Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae, and α-hemolysin from the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. *FEMS Microbiol. Lett*. **126,** 189–195.

Carmona, M., Balsalobre, C., Munoa, F.J., Mourino, M., Jubete, Y., De la Cruz, F. and Juarez, A. (1993). *Escherichia coli hha* mutants, DNA supercoiling, and expression of the hemolysin genes from the recombinant plasmid pANN202-312. *Mol. Microbiol*. **9,** 1011–1018.

Clementz, T., Zhou, Z.M. and Raetz, C.R.H. (1997). Function of the *Escherichia coli msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipidA: acylation of MsbB follows laurate incorporation by HtrB. *J. Biol. Chem*. **272,** 10353–10360.

Comayras, C., Tasca, C., Peres, S.Y., Ducommun, B., Oswald, E. and De Rycke, J. (1997). *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G2/M transition by preventing cdc2 protein kinase dephosphorylation and activation. *Infect. Immun*. **65,** 5088–5095.

Cornelis, G.R., Sluiters, C., Delor, I., Gelb, D., Kaninga, K., Lambert de Rouvroit, C., Sory, M.P., Vanooteghem, J.C. and Michaelis (1991). *ymoA*, a *Yersinia enterocolitica* chromosomal gene modulating expression of virulence functions. *Mol. Microbiol*. **5,** 1023–1034.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2001). Glycophorin as a receptor for *Escherichia coli* α-hemolysin in erythorcytes. *J. Biol. Chem*. **276,** 12513–12519.

Cortajarena, A.L., Goni, F.M. and Ostolaza, H. (2003). A receptor-binding region in *Escherichia coli* alpha-hemolysin. *J. Biol. Chem*. **278,** 19159–19163.

De Rycke, J., Gonzales, E.A., Blanco, J., Oswald, Blanco, M. and Boivin, R. (1990). Evidence for two types of cytotoxic necrotizing factors in human and animal clinical isolates of *Escherichia coli*. *J. Clin. Microbiol*. **28,** 694–699.

Dutta, P.R., Cappello, R., Navarro-Garcia, F. and Ntaro, J.P. (2002). Functional comparison of serine protease autotransporters of *Enterobacteriaceae*. *Infect. Immun*. **70,** 7105–7113.

Forestier, C. and Welch, R.A. (1990). Non-reciprocal complementation of the *hlyC* and *lktC* genes of the *Escherichia coli* hemolysin and *Pasteurella haemolytica* leukotoxin determinants. *Infect. Immun*. **58,** 828–832.

Gentschev, I., Dietrich, G. and Goebel, W. (2002). The *E. coli* α-hemolysin secretion system and its use in vaccine development. *TRENDS Microbiol*. **10,** 39–45.

Gentschev, I. and Goebel, W. (1992). Topological and functional studies on HlyB of *Escherichia coli*. *Mol. Gen. Genet*. **232,** 40–48.

Guyer, D.M., Kao, J.S. and Mobley, H.L. (1998). Genomic analysis of a pathogenicity island in uropathogenic *Escherichia coli* CFT073: distribution of homologous sequences among isolates from patients with pyelonephritis, cystitis, and catheter-associated bacteriuria and from fecal samples. *Infect. Immun*. **66,** 4411–4417.

Guyer, D.M., Henderson, I.R., Nataro, J.P. and Mobley, H.L. (2000). Identification of Sat, an autotransporter toxin produced by uropathogenic *Escherichia coli*. *Mol. Microbiol*. **38,** 53–66.

Gygi, D., Nicolet, J., Frey, J., Cross, M., Koronakis, V. and Hughes, C. (1990). Isolation of the *Actinobacillus pleuropneumoniae* hemolysin gene and the activation and secretion of the prohemolysin by the HlyC, HlyB, and HlyD proteins of *Escherichia coli*. *Mol. Microbiol*. **4,** 123–128.

Hacker, J. and Hughes, C. (1985). Genetics of *Escherichia coli* hemolysin. *Curr. Top. Microbiol. Immunol*. **118,** 139–162.

Henderson, B., Poole, S. and Wilson, M. (1996). Bacterial modulins—a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev*. **60,** 316–341.

Holland, I.B. and Blight, M.A. (1999). ABC-ATPases, adaptable energy generators fueling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J. Mol. Biol*. **293,** 381–399.

Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertury, A., LeCaer, J.P., Danchin, A. and Bertin, P. (2001). Large scale monitoring of pleiotropic regulation of gene expression by the prokaryotic nucleoid-associated protein H-NS. *Mol. Microbiol*. **40,** 20–36.

Issartel, J.P., Koronakis, V. and Hughes, C. (1991). Activation of *Escherichia coli* prohemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature* **351,** 750–761.

Jarchau, T., Chakraborty, T., Garcia, F. and Goebel, W. (1994). Selection for transport competence of C-terminal polypeptides derived from *Escherichia coli* hemolysin: the shortest peptide capable of autonomous HlyB/HlyD-dependent secretion comprises the C-terminal 62 amino acids of HlyA. *Mol. Gen. Genet*. **245,** 53–60.

Johnson, J.M. and Church, G.M. (1999). Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J. Mol. Biol*. **287,** 695–715.

Kao, J.S., Stucker, D.M., Warren, J.W. and Mobley, H.L. (1997). Pathogenicity island sequences of pyelonephritogenic *Escherichia coli* CFT073 are associated with virulent uropathogenic strains. *Infect. Immun*. **65,** 2812–2820.

Knapp, S., Hacker, J., Jarchau, T. and Goebel, W. (1986). Large, unstable inserts in the chromosome affect virulence properties of uropathogenic *Escherichia coli* O6 strain 536. *J. Bactériol*. **168,** 22–30.

Koronakis, V., Sharff, A., Koronakis, E., Luisi, B. and Hughes, C. (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405,** 914–919.

Koronakis, V., Cross, M., Senior, B., Koronakis, E. and Hughes, C. (1987). The secreted hemolysins of *Proteus mirabilis, Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. *J. Bacteriol*. **169,** 1509–1515.

Koronakis, E., Hughes, C., Milisav, I. and Koronakis, V. (1995). Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HlyB. *Mol. Microbiol*. **16,** 87–96.

Koronakis, V. and Hughes, C. (1996). Synthesis, maturation, and export of the *Escherichia coli* hemolysin. *Med. Microbiol*. **23,** 65–71.

Lilie, H., Haehnel, W., Rudolph, R. and Baumann, U. (2000). Folding of a synthetic parallel beta-roll protein. *FEBS Lett*. **470,** 173–177.

Ludwig, A., von Rhein, C., Bauer, S., Huttinger, C. and Goebel, W. (2004). Molecular analysis of cytolysin A (ClyA) in pathogenic *Escherichia coli* strains. *J. Bacteriol*. **186,** 5311–5320.

Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J. and Goebel, W. (1996). Analysis of the *in vivo* activation of hemolysin (HlyA) from *Escherichia coli*. *J. Bacteriol*. **178,** 5442–5430.

Ludwig, A., Jarchau, T., Benz, R. and Goebel, W. (1988). The repeat domain of *Escherichia coli* hemolysin (HlyA) is responsible for its $Ca^{2+}$-dependent binding to erythrocytes. *Mol. Gen. Genet*. **214,** 553–561.

Ludwig, A., Schmid, A., Benz, R. and Goebel, W. (1991). Mutations affecting pore formation by hemolysin from *Escherichia coli*. *Mol. Gen. Genet*. **226,** 198–208.

Ludwig, A., Vogel, M. and Goebel, W. (1987). Mutations affecting activity and transport of hemolysin in *Escherichia coli*. *Mol. Gen. Genet*. **206,** 238–245.

Madrid, C., Nieto, J.M., Paytubi, S., Falconi, M., Gualerzi, C.O. and Juarez, A. (2002). Temperature- and H-NS-dependent regulation of a plasmid-encoded virulence operon expressing *Escherichia coli* hemolysin. *J. Bacteriol*. **184,** 5058–5066.

Menestrina, G., Pederzolli, C., Dalla Serra, M., Bregante, M. and Gambale, F. (1996). Permeability increases induced by *Escherichia coli* hemolysin A in human macrophages is due to the formation of ionic pores: a patch clamp characterization. *J. Membr. Biol*. **149,** 113–121.

Middendorf, B., Hochhut, B., Leipold, K., Dobrindt, U., Blum-Oehler, G. and Hacker, J. (2004). Instability of pathogenicity islands in uropathogenic *Escherichia coli* 536. *J. Bacteriol*. **186,** 3086–3096.

Mourino, M., Madrid, C., Balsalobre, C., Prenafeta, A., Munoa, F., Blanco, J., Blanco, M., Blanco, J.E. and Juarez, A. (1996). The Hha protein as a modulator of expression of virulence factors in *Escherichia coli. Infect. Immun*. **64,** 2881–2884.

Mourino, M., Balsalobre, C., Madrid, C., Nieto, J.M., Prenafeta, A., Munoa, F.J. and Juarez, A. (1998). Osmolarity modulates the expression of the Hha protein from *Escherichia coli. FEMS Microbiol. Lett*. **160,** 225–229.

Nagy, G., Dobrindt, U., Blum-Oehler, G., Emödy, L., Goebel, W. and Hacker, J. (2000). Analysis of the hemolysin determinants of the uropathogenic *Escherichia coli* strain 536. *Adv. Exp. Med. Biol*. **485,** 57–61.

Nieto, J.M., Bailey, M.J.A., Hughes, C. and Koronakis, V. (1996). Supression of transcription polarity in the *Escherichia coli* hemolysin operon by a short upstream element shared by polysaccharide and DNA transfer determinants. *Mol. Microbiol*. **19,** 705–713.

Nieto, J.M., Madrid, C., Prenafeta, A., Miquelay, E., Balslobre, C., Carrascal, M. and Juarez, A. (2000). Expression of the hemolysin operon in *Escherichia coli* is modulated by a nucleo-protein complex that includes the proteins Hha and H-NS. *Mol. Gen. Genet*. **263,** 349–358.

Noegel, A., Rdest, U. and Goebel, W. (1981). Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli. J. Bacteriol*. **145,** 233–247.

Ostolaza, H., Soloaga, A. and Goni, F.M. (1995). The binding of divalent cations to *Escherichia coli a*-hemolysin. *Eur. J. Biochem*. **228,** 39–44.

Oswald, E., De Rycke, J., Guillot, J.F. and Boivin, R. (1989). Cytotoxic effect of multinucleation in HeLa cell cultures associated with the presence of Vir plasmid in *Escherichia coli* strains. *FEMS Microbiol. Lett*. **58,** 95–100.

Parham, N.J., Srinivasan, U., Desvaux, M., Foxman, B., Marrs, C.F. and Henderson, I.R. (2004). PicU, a scond serine protease autotransporter of uropathogenic *Escherichia coli. FEMS Microbiol. Lett*. **230,** 73–83.

Paulsen, I.T., Park, J.H., Choi, P.S. and Saier, M.N. Jr. (1997). A family of Gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs, and heavy metals from Gram-negative bacteria. *FEMS Microbiol. Lett*. **156,** 1–8.

Rippere-Lampe, K.E., O'Brien, A.D., Conran, R. and Lockman, H.A. (2001). Mutation of the gene encoding cytotoxic necrotizing factor type 1 (*cnf1*) attenuates the virulence of uropathogenic *Escherichia coli. Infect. Immun*. **69,** 3954–3964.

Rowe, G.E., Pellett, S. and Welch, R.A. (1994). Analysis of toxinogenic functions associated with the RTX repeat region and monoclonal antibody D12 epitope of *Escherichia coli* hemolysin. *Infect. Immun*. **62,** 579–588.

Schlor, S., Schmidt, A., Maier, E., Benz, R., Goebel, W. and Gentschev, I. (1997). *In vivo* and *in vitro* studies on interactions between the components of the hemolysin (HlyA) secretion machinery of *Escherichia coli. Mol. Gen. Genet*. **256,** 306–319.

Schmidt, H., Kernbach, C. and Karch, H. (1996). Analysis of the EHEC *hly* operon and its location in the physical map of the large plasmid of enterohemorrhagic *Escherichia coli* O157:H7. *Microbiol*. **142,** 907–914.

Schülein, R., Gentschev, I., Mollenkopf, H.J. and Goebel, W. (1992). A topological model for the hemolysin translocator protein HlyD. *Mol. Gen. Genet*. **234,** 155–163.

Scott, D.A. and Kaper, J. (1994). Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. *Infect. Immun*. **62,** 244–251.

Soloaga, A., Veiga, M.P., Garcia-Segura, L.M., Ostolaza, H., Brasseur, R. and Goni, F.M. (1999). Insertion of *Escherichia coli* α-hemolysin in lipid bilayers as a non-transmembrane integral protein: prediction and experiment. *Mol. Microbiol*. **31,** 1013–1924.

Stanley, P., Packman, L.C., Koronakis, V. and Hughes, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science* **266,** 1992–1996.

Stanley, P., Koronakis, V., Hardie, K. and Hughes, C. (1996). Independent interaction of the acyltransferase HlyC with two maturation domains of *Escherichia coli* toxin HlyA. *Mol. Microbiol*. **20,** 813–822.

Stanley, P., Koronakis, V. and Hughes, C. (1998). Acylation of *Escherichia coli* hemolysin: a unique protein lipidation mechanism underlying toxin function. *Microbiol. Mol. Biol. Rev*. **62,** 309–333.

Strathdee, C.A. and Lo, R.Y.C. (1987). Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha-hemolysin of *Escherichia coli. Infect. Immun*. **55,** 3233–3236.

Swenson, D.L., Bukanov, N.O., Berg, D.E. and Welch, R.A. (1996). Two pathogenicity islands in uropathogenic *Escherichia coli* J96: cosmid cloning and sample sequencing. *Infect. Immun*. **64,** 3736–3743.

Uhlen, P., Laestadius, A., Jahnukainen, T., Söderblom, T., Bäckhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A. and Richter-Dahlfors, A. (2000). α-hemolysin of uropathogenic *E. coli* induces $Ca^{2+}$ oscillations in renal epithelial cells. *Nature* **405,** 694–697.

Vogel, M., Hess, J., Then, I., Juarez, A. and Goebel, W. (1988). Characterization of a sequence (*hlyR*) which enhances synthesis and secretion of hemolysin in *Escherichia coli. Mol. Gen. Genet*. **212,** 76–84.

Wagner, W., Vogel, M. and Goebel, W. (1983). Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bacteriol*. **154,** 200–210.

Walev, I., Vollmer, P., Palmer, M., Bhakdi, S. and Rose-John, S. (1996). Pore-forming toxins trigger shedding of receptors for interleukin 6 and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **93,** 7882–7887.

Welch, R.A., Bauer, M.E., Kent, A.D., Leeds, J.A., Moayeri, M., Regassa, L.B. and Swenson, D.L. (1995). Battling against host phagocytes: the wherefore of the RTX family of toxins? *Infect. Agents. Dis*. **4,** 254–272.

Welch, R.A. and Pellet, S. (1988). Transcriptional organization of the *Escherichia coli* hemolysin genes. *J. Bacteriol*. **170,** 1622–1630.

Zgurskaya, H.L. and Nikaido, H. (2000). Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol*. **37,** 219–225.

CHAPTER

# 48

# *Escherichia coli*, *Vibrio*, and *Yersinia* species heat-stable enterotoxins

*J. Daniel Dubreuil*

## INTRODUCTION

Pathogenesis manifestation of enteric pathogens represents a complex and coordinated expression of several virulence factors. These factors enable the bacterial pathogen to breach host defenses to effectively colonize its niche. Among many proteins elaborated during such processes, the heat-stable enterotoxins (STs) are important molecules involved in the diarrhea resulting from such infections in humans and animals. The heat-stable toxins comprise a family of cysteine-rich peptides elaborated by various bacterial species, of whom many are more or less related structurally and functionally. Diarrhea induced by STs is of the secretory type involving the small intestine with no signs of inflammation.

ST was first described in enterotoxigenic *Escherichia coli* (ETEC). Based on structure-function, the ETEC STs are divided into two distinct types, namely STa (or STI) and STb (or STII) (Burgess *et al*., 1978; Weikel and Guerrant, 1985). STa differs from STb by its resistance to proteases, its solubility in methanol, and its activity in the suckling-mouse model. On the other hand, STb is particularly sensitive to proteolysis, insoluble in methanol, and was shown to be active solely in weaned piglets. Addition of protease inhibitors to the tested sample was later shown to render other animals (i.e., rats, mice, rabbits, and calves) sensitive to STb (Whipp, 1990, 1991). In enteroaggregative *E. coli* (EAEC), a heat-stable toxin was identified. This toxin coined EAST1 has been shown to cause diarrhea principally in humans. EAST1 is often compared to STa as it shows structural similarities, as well as an increase in the synthesis of the same signaling molecule.

Apart from *E. coli*, other enteric bacteria produce ST toxins (Figure 48.1). These include strains from *Vibrio cholerae* O1 producing O1-ST (Takeda *et al*., 1991a; Mallard and Desmarchelier, 1995), *V. cholerae* non-O1 or non-O139 serotypes producing NAG-ST (Takao *et al*., 1985a; Arita *et al*., 1986), H-ST from Hakata strains of *V. cholerae* non-O1 (Arita *et al*., 1991a), and *Vibrio mimicus* producing M-ST (Arita *et al*., 1991b; Yuan *et al*., 1994; Ramamurthy *et al*., 1994). *Yersinia enterolitica* produces Y-ST with subtypes Y-STa, Y-STb, and Y-STc (Takao *et al*., 1984; Delor *et al*., 1990; Huang *et al*., 1997; Ramamurthy *et al*., 1997). In addition, Yk-ST is elaborated by *Yersinia kristensenii* (Delor *et al*., 1990; Ibrahim *et al*., 1992), C-ST from *Citrobacter freundii* (Guarino *et al*., 1987a, 1989a), and ST-like toxin by *Klebsiella pneumoniae* (Guarino *et al*., 1989b).

## DISEASES CAUSED BY *E. COLI*, *YERSINIA*, AND *VIBRIO*

ETEC are a major cause of diarrheal disease in humans and animals. They are responsible for diarrhea by production of two distinct types of enterotoxin: the heat-labile (LT) toxin with two subtypes (LTI and LTII) and a family of heat-stable (ST) enterotoxins. LT is a high-molecular-mass toxin (85 kDa) with two subtypes (LTI and LTII) functionally and structurally related to

| Molecules | Number of amino acids | $NH_2$-terminus | COOH-terminus | References |
|---|---|---|---|---|
| ***Escherichia coli* STaH** | 19 | N–S–S–N–Y–**C**–**C**–E–L–**C**–**C**– | **N**–**P**–**A**–**C**–T–G–C–Y | Aimoto *et al.* (1982) |
| ***Escherichia coli* STaP** | 18 | N–T–F–Y–**C**–**C**–E–L–**C**–**C**– | **N**–**P**–**A**–**C**–A–G–C–Y | Takao *et al.* (1983) |
| ***Escherichia coli* EAST1** | | | | |
| **17-2** | 38 | ...A–S–S–Y–A–S–**C**–I–W–**C**–T—— | T–**A**–**C**–A–S–C–H–G ... | Savarino *et al.* (1993) |
| **0-42** | 38 | ...A–S–S–Y–A–S–**C**–I–W–**C**–A—— | T–**A**–**C**–A–S–C–H–G ... | Yamamoto *et al.* (1997) |
| ***Vibrio cholerae* 01** | | | | |
| **01ST-1** | 17 | I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Yoshino *et al.* (1993) |
| **01ST-2** | 18 | L–I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Yoshino *et al.* (1993) |
| **01ST-3** | 19 | N–L–I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Yoshino *et al.* (1993) |
| **01ST-4** | 28 | ...N–L–I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Yoshino *et al.* (1993) |
| ***Vibrio cholerae*** | | | | |
| **non-01(NAG-ST)** | 17 | I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Yoshimura *et al.* (1986) |
| ***Vibrio cholerae* non-01 (H-ST)** | 18 | L–I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Arita *et al.* (1991a) |
| Hakata strain | | | | |
| ***Vibrio mimicus* (M-ST)** | 17 | I–D–**C**–**C**–E–I–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C–L–N | Arita *et al.* (1991a) |
| ***Yersinia enterolitica* Y-STa** | 30 | ...S–S–D–W–D–**C**–**C**–D–V–**C**–**C**– | **N**–**P**–**A**–**C**–A–G–C | Takao *et al.* (1985) |
| **Y-STb** | 30 | ...E–E–N–D–D–W–**C**–**C**–E–V–**C**–**C**– | **N**–**P**–**A**–**C**–A–G–C | Yoshino *et al.* (1994) |
| **Y-STc** | 53 | ...E–N–D–W–D–W–**C**–**C**–E–L–**C**–**C**– | **N**–**P**–**A**–**C**–F–G–C | Yoshino *et al.* (1995) |
| ***Citrobacter*** | | | | |
| ***freundii* (C-ST)** | 18 | N–T–F–Y–**C**–**C**–E–L–**C**–**C**– | **N**–**P**–**A**–**C**–A–G–C–Y | Guarino *et al.* (1989) |
| ***Conus geographus*** | 13 | E–**C**–**C**– | **N**–**P**–**A**–**C**–G–R–H–Y–S–C | Gray *et al.* (1981) |
| $\alpha$-conotoxin $G_I$ | | | | |
| **Guanylin** (Human) | 15 | P–G–T–**C**–E–I–**C**–A–Y–A– | **A**–**C**–T–G–C | Greenberg *et al.* (1997) |
| (Rat) | 15 | P–N–T–**C**–E–I–**C**–A–Y–A– | **A**–**C**–T–G–C | Greenberg *et al.* (1997) |
| **Uroguanylin** (Human) | 15 | N–D–D–**C**–E–L–**C**–V– | **N**–V–**A**–**C**–T–G–C–L | Greenberg *et al.* (1997) |
| (Rat) | 15 | T–D–E–**C**–E–L–**C**–I– | **N**–V–**A**–**C**–T–G–C | Greenberg *et al.* (1997) |

**FIGURE 48.1** Primary sequences of *Escherichia coli* STaH and STaP compared to other heat-stable enterotoxins of bacterial origins (including *Vibrio*, *Yersinia*, and *Citrobacter*). The sequence *of Conus geographus*, $\alpha$-conotoxin $G_I$, is also represented as it demonstrates some homology to STa. Guanylin and uroguanylin sequences of human and rat origin are also aligned for comparison purposes. The number of amino acids comprised in the mature molecules is indicated. In bold are represented the consensus sequence found in most heat-stable toxins. In the box, the consensus N-P-A-C sequence conserved at least partially between all the molecules appearing in the figure.

*V. cholerae* enterotoxin (Sprangler, 1992). STs of two types are produced (STa and STb). Some strains may produce one or both of these types of toxins. Colonization of the intestinal mucosa allows for the localized delivery of these enterotoxins. The biological activities lead to net fluid secretion occurring in the jejunum and ileum, with a greater fluid loss in the jejunum resulting in a watery diarrhea characteristic of ETEC infection. About 46% of ETEC isolates express STa alone, 25% express LT alone, and 29% express both STa and LT (Wolf, 1997). EAST1 originally isolated from an EAEC strain has been proposed to be implicated in the pathogenesis of EAEC leading to diarrhea. EAEC strains harboring *astA* gene, coding for EAST1 toxin, as the only identified virulence factor have been associated with outbreaks and sporadic cases of diarrhea (Itoh *et al*., 1997; Kawano *et al*., 1998; Zhou *et al*., 2002). EAST1 was proposed as another possible cause of traveler's disease (Gascon *et al*., 1998; Vila *et al*., 2000).

*V. cholerae* is the causative agent of cholera. Of the numerous serotypes, only serotypes O1 and 139 are associated with the disease known as cholera. The other serotypes known as non-O1 non-O139 are recognized as the etiological agents of gastroenteritis and some extraintestinal infections. Sometimes, the clinical features of diarrhea caused by non-O1 non-O139 serotypes cannot be distinguished from cholera (Morris *et al*., 1990). Some strains of *V. cholerae* O1, V. *cholerae* non-O1 (Hakata strains), and *V. mimicus* produce ST and have been implicated in diarrheal disease (Takeda *et al*., 1991a; Arita *et al*., 1991a; Ramamurthy *et al*., 1994). *V. mimicus*, another human pathogen, produces a ST responsible for cases of diarrhea. It was determined that *V. mimicus* is the reservoir of the ST gene (*stn*) (Yuan *et al*., 1994).

*Yersinia enterolitica* is a well-established enteric pathogen capable of causing a variety of clinical disorders in humans. While diarrhea is the most commonly recognized clinical manifestation, it can also be responsible for invasive diseases like mesenteric lymphoadenitis, ileitis, and septicemia. It can also be associated with various autoimmune manifestations, including

arthritis and erythrema nodosum (Cover and Aber, 1989). Production of Y-ST by *Y. enterolitica* is postulated to be important in the pathogenesis of watery diarrhea. Its production depends on culture medium, temperature, and growth phase (Boyce *et al*., 1979; Delor *et al*., 1990; Amirmozafari and Robertson, 1993). In commonly used culture media, Y-ST is detected only when the temperature is below 30˚C. This finding was difficult to reconcile with diarrhea sustained for long periods at body temperature. We now know that production of Y-ST can be observed at 37°C, when the osmolarity and pH of the culture medium are similar to those of the intestinal environment (Mikulskis *et al*., 1994). The biological expression of Y-ST is detected using the suckling mouse assay (Pai and Mors, 1978).

## *E. COLI* STa ENTEROTOXIN

### STa polypeptide

STa represents a family of toxins of approximately 2,000 Da composed of a single peptide chain (Dreyfus *et al*., 1983; Thompson and Gianella, 1985). Toxins produced by human (STaH) and porcine (STaP) strains differ slightly. The STaH primary structure comprises 19 amino acid residues compared with 18 for STaP. The amino acid sequences of the two toxin subtypes are not identical (Figure 48.1). To date, STaP polypeptide has been observed in isolates from animals species including pigs, calves, lambs, chickens, and horses and also from humans. In contrast, STaH is produced solely by human isolates. These toxins share a highly conserved sequence of 15 amino acid residues, which reside in the carboxy-terminus. This sequence represents a common antigenic determinant. The amino-terminal amino acids to the first cysteine can be cleaved from the molecule and the remaining peptide is fully active (Staples *et al*., 1980).

Both toxins are synthesized as larger precursors (propre-STa) that are subsequently cleaved to the active mature toxin. Six cysteine residues involved in disulfide bond formation are present at the same position in both STaH and STaP (Figure 48.2). The tertiary structure formed by the disulfide bonds is critical and required for full biological activity (Okamoto *et al*, 1987). Chemically synthesized STa behaves in a similar manner to ETEC-produced STa (Gyles, 1994; Takeda *et al*., 1991b).

Native STa toxins are poorly immunogenic. However, they can be conjugated to carrier proteins in order to produce polyclonal antisera and monoclonal antibodies (Aitken and Hirst, 1993; Brandwein *et al*., 1985). The toxicity of STaH and STaP can be neutralized by homologous but also by heterologous antisera (Takeda *et al*., 1983).

### Biochemical characteristics

STa enterotoxin is a heat-stable molecule, and its small size is responsible for this biochemical characteristic. It is an acidic peptide with a pI of 3.98 (Dreyfus *et al*., 1983). The molecule is soluble in water and organic solvents, including methanol, and resistant to several proteases, such as pronase, trypsin, and chymotrypsin. It is resistant to acidic but not to basic pH. The toxin is completely inactivated by reducing and oxidizing agents, which disrupt disulfide bonds (Dreyfus *et al*., 1983).

### *estA* gene

The genes encoding STa are found on plasmids of varying molecular sizes (Harnett and Gyles, 1985). For animal ETEC isolates, it is common to find gene coding for STa, colonization factor, drug resistance, and production of colicin on the same plasmid. For human isolates, the same plasmid was shown to carry *estA* and an antibiotic resistant gene (Gyles *et al*., 1977; Echeverria *et al*., 1978). Genetic studies demonstrated the existence of two types of *estA*. The gene was first cloned from a bovine isolate (STaP) and shown to be part of a transposon (Tn *1681*) that is flanked by inverted repeats of IS*1* (So and McCarthy, 1980). STaP genes from ETEC isolated from other animal species (including humans) are also part of the same transposon (Sekizaki *et al*., 1985). Genes encoding STaH and STaP may be carried by a single human ETEC strain. Synthesis of STa by *E. coli* is subject to catabolite repression, and optimal yields of toxin are obtained in glucose-free media (Alderete and Robertson, 1997a, 1977b; Stieglitz *et al*., 1988). A study by Sommerfelt *et al*. (1989) revealed that loss of STa production is the result of deletions of DNA fragments harboring the toxin gene rather than loss of plasmids.

### Secretion of STa and formation of disulfide bonds

STa is produced as a 72 amino acid precursor molecule referred to as pre-pro-STa (Stieglitz *et al*., 1988; Okamoto and Takahara, 1990). The 72 amino acid polypeptide consists of a 19 amino acid signal peptide (pre-STa), a 35 amino acid pro sequence, and then an 18 or 19 amino acid mature STa (Figure 48.2B). Whereas the pro-STa is translocated accross the cytoplasmic membrane and seems to require *secA*-dependent transport, the signal sequence is cleaved by peptidase 1 (Rasheed *et al*., 1990). Yamanaka and Okamoto (1990) subsituted the charged amino acids at positions 29 to 31 of the pro-region of STa. Hydrophobic or basic residues significantly reduced STa translocation across

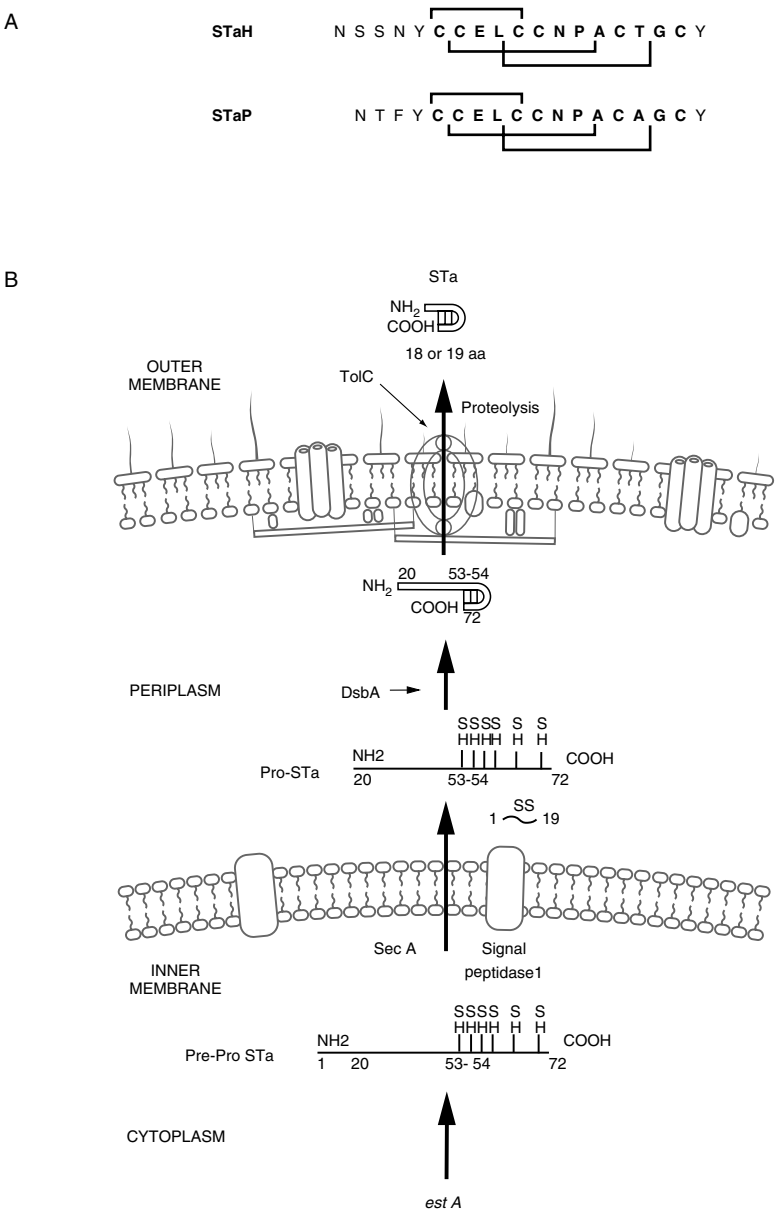



**FIGURE 48.2** A) Primary sequences of STaH and STaP showing the disulfide bond structure. In bold, the 13 amino acid residues constituting the toxic domain. B) Disulfide bonds formation and secretory pathways, as determined for STa toxin.

the inner membrane, indicating that a net negative charge near amino acid 30 is important for efficient translocation. The pro-region was shown to guide STa into the periplasmic space (Yamanaka *et al*., 1993). However, this region does not seem to be involved in transport of the peptide extracellularly (Okamoto and Takahara, 1990).

In the periplasm, the three intramolecular disulfide bonds, important for toxicity, are formed by DsbA protein prior to secretion (Okamoto *et al*., 1995; Yang *et al*., 1992; Sanchez *et al*., 1993). In the pro-region, the cysteine-39 residue is important for the correct recognition by DsbA and proteases during maturation of STa (Yamanaka *et al*., 1994). A negative charge at position 7 in mature STaP (Glu) was shown to be required for toxin interaction with DsbA in the periplasm and formation of the intramolecular disulfide bonds *in vivo* (Yamanaka *et al*., 1998b).

A second proteolysis event occurs extracellularly, owing to an undefined protease, to produce biologically active 18 and 19 amino acids STa (Rasheed *et al*., 1990). STa toxin is secreted from the cell as it is synthesized and is not cell-associated. Mature STa diffuses across the outer membrane (Yamanaka *et al*., 1994; Yang *et al*., 1992). TolC is involved in the crossing of the outer membrane as STa was not secreted by a TolC-deficient strain (Yamanaka *et al*., 1998a). Yamanaka *et al*. (1997) demonstrated, by mutating every cysteine

residue of STa, that the formation of the three intramolecular disulfide bonds is not absolutely necessary for the mature toxin to pass through the outer membrane.

The three intramolecular disulfide bonds in STaH link cysteines 6 and 11, 7 and 15, and 10 and 18; in STaP disulfide bonds link cysteines 5 and 10, 6 and 14, and 9 and 17 (Figure 48.2A). The use of STa analogues demonstrated that the second disulfide bond was essential for toxicity, but analogues lacking the first or the third disulfide bond showed only a reduced toxicity (Hidaka *et al*., 1991). In conclusion, the disulfide bonds between cysteines 7 and 15 of STaH or 6 and 14 of STaP are essential for biological activity.

## Structure of STa and identification of the toxic domain

The three-dimensional structure of STa was determined using nuclear magnetic resonance spectroscopy and x-ray crystallography. The tertiary structure consists of a folded peptide backbone assembled as a right-handed spiral from the first cysteine at the $NH_2$-terminus to the last cysteine residue at the COOH-terminus (Ozaki *et al*., 1991). Three β-turns are located along this spiral and are stabilized by the three intramolecular disulfide bonds (Gariépy *et al*., 1986, 1987). The disulfide bonds are responsible for the spatial structure of STa and this 3D structure is required for toxicity. The STa molecule appears to be hydrophobic, but there are three hydrophilic areas (i.e., $Cys^6$–$Leu^8$, $Cys^{10}$–$Asp^{11}$, and $Pro^{12}$–$Cys^{17}$) in STaP (Ozaki *et al*., 1991).

Overall, a 13 amino acid sequence from the amino-terminal cysteine to the carboxyl-terminal cysteine is essential for toxic activity of both STaH and STaP (Yoshimura *et al*., 1985; Carpick and Gariépy, 1991; Waldman and O'Hanley, 1989). Thus, this segment constitutes the toxic domain of STa (Figure 48.2A). For this region, a striking identity is shared with other heat-stable enterotoxins secreted by other enteric pathogens such as *Citrobacter freundii, Yersinia enterolitica, Vibrio cholerae* O1 and non-O1 strains, and *Vibrio mimicus* (Figure 48.1). The second β-turn at residues 11 to 14 (STaP) is proposed to be the most important for toxicity of STa, as this region of the molecule directly interacts with its receptor (Ozaki *et al*., 1991; Sato *et al*., 1994). More specifically, alanine at position 13 in STaP and at position 14 in STaH is essential for toxicity. The methyl side chain of this alanine is directed outward of the molecule and could interact directly with a hydrophobic area located on the surface of the receptor.

The four amino acids (N-P-A-C) are conserved in STaP and STaH and other heat-stable enterotoxins produced by other bacterial species (Figure 48.1). This sequence is also conserved in the marine snail *Conus geographus,* α-conotoxin $G_I$. In guanylin and *E. coli* EAST-1 toxin, an alanine followed by a cysteine residue is partially conserved (i.e., X-X-A-C). For human and rat uroguanylin, the conserved sequence is N-X-A-C. It is interesting to note that in guanylin, the endogeneous ligand for STa receptor, these amino acids interact with the receptor (Greenberg *et al*., 1997).

## Receptor identity

Since the mid-1980s, numerous studies have demonstrated that STa can bind non-covalently, with high or low affinity, to several proteins in the plasma membrane of susceptible eucaryotic cells (Dreyfus and Robertson, 1984b; Waldman *et al*., 1986).

STa stimulates particulate guanylate cyclase in intestinal epithelial cells, resulting in elevation of cyclic GMP (cGMP) (Field *et al*., 1978; Hughes *et al*., 1978). The same studies also demonstrated that 8-bromo-cGMP mimics the effect of STa in animal systems. Thus, the activity of STa is mediated by the intracellular second messager cGMP and research was focused on evaluating binding of STa to cellular proteins and on demonstration of the possible guanylate cyclase activity of the molecule.

The STa receptor has been the subject of intensive studies. Several solubilization and cross-linking experiments using $^{125}$I-STa and intestinal brush border membranes aimed at identifying the STa receptor were carried out. For example, de Sauvage *et al*. (1992) have identified proteins of 49, 56, 68, 81, 133 and 153 kDa, whereas Hirayama *et al*. (1992) identified a 200-kDa protein cross-linked to radioiodinated STa. Purification of the receptor by ligand-affinity chromatography identified a protein subunit of 74 kDa to which $^{125}$I-STa was binding (Hughes *et al*., 1992). The molecules identified in these studies were glycoproteins, but none showed guanylate cyclase activity as expected. The heterogeneity of the proposed intestinal receptors for STa is exemplified by additional studies (Thompson and Gianella, 1990; Ivens *et al*., 1990; Hakki *et al*., 1993; Cohen *et al*., 1993; Hughes *et al*., 1991).

Conclusive identification of STa receptor was achieved when the STa receptor was cloned from cDNA libraries of rat (Schulz *et al*., 1990), pig (Wada *et al*., 1994), and human intestine (de Sauvage *et al*., 1991). New members of the guanylyl cyclase family were looked for in the small intestinal mucosa using degenerated oligonucleotide primers based on conserved sequences in the catalytic domains of membrane and soluble guanylyl cyclases. When the cDNA was transfected into naive COS cells, specific STa binding activity and guanylate cyclase activity were expressed (Schulz *et al*., 1990). The deduced amino acid

sequence and functional expression in mammalian cells indicated that the STa receptor was guanylyl cyclase C (GC-C) belonging to the atrial natriuretic peptide receptor family that included GC-A and GC-B. (Chang *et al*., 1989; Hirayama *et al*., 1992; Vaandrager *et al*., 1993a, 1993b). For these receptors, the binding site and the catalytic activity of guanylate cyclase resided on the same protein.

The GC-C unglycosylated protein has a Mr of 120 kDa and it is found as a 140- to 160-kDa glycoprotein after N-linked glycosylation (Mann *et al*., 1993). At the $NH_2$-terminus, it consists of an extracellular receptor domain, a transmembrane domain, and cytoplasmic domain, including a kinase homology domain and a guanylyl cyclase catalytic domain at the COOH-terminus. GC-C null mice are refractory to the secretory action of STa, proving that the GC-C receptor is necessary for the diarrheal response induced by this toxin (Mann *et al*, 1997).

Thus, STa activates particulate guanylate cyclase. Particulate guanylate cyclases are brush border membrane glycoproteins. In the human intestine, approximately 75–80% of the total guanylate cyclase activity is particulate; the rest is soluble (Garbers *et al*, 1991; Schulz *et al*, 1991). GC-C also contains a carboxy-terminal tail of about 60 amino acids. The function of this segment is uncertain, but possibly links the molecule to the cytoskeleton. The endogeneous agonist for GC-C was later found to be a 15-amino acid hormone called guanylin (Greenberg *et al*., 1997). This hormone appears to play a role in the regulation of fluid and electrolyte absorption in the gut. Guanylin is 50% homologous to STa and it contains 4 cysteine residues that are involved in disulfide bond formation and are essential for biological activity (Figure 48.1). This hormone is less potent than STa in activating GC-C and in stimulating chloride secretion ($Cl^-$) (Currie *et al*., 1992; Wiegand *et al*., 1992; Forte *et al*., 1993; Carpick and Gariépy, 1993; Kuhn *et al*., 1994; Greenberg *et al*., 1997). It thus appears that STa opportunistically utilizes GC-C to alter the basal gut homeostasis.

A form of guanylin circulates in the blood of patients with chronic kidney failure (Kuhn *et al*, 1993). There also exists in animal and human urine a guanylin-like molecule called uroguanylin (Hamra *et al*., 1993; Kita *et al*., 1994). It is clear from their sequences that guanylin and uroguanylin are the products of separate genes. It thus appears that guanylin but also uroguanylin are endogeneous ligands for GC-C and could be modulators of $Cl^-$ secretion in the intestine, and also in kidneys and other organs (Giannella, 1995; Greenberg *et al*., 1997). The receptor for these molecules is located on enterocytes, colonocytes, and cells of various extraintestinal tissues.

## *In vitro* studies involving cell lines and *in vivo* analysis of the receptor distribution

Intestinal cell lines were used to study the *in vitro* binding of STa and its biological action. For example, both human cancer cell lines Caco-2 and T84 possess the specific receptor for STa (Guarino *et al*., 1987b; Cohen *et al*., 1993). Binding of STa to these cell lines is coupled to cGMP production as was previously observed in the rat. When T84 polarized cells are mounted in a Ussing chamber they can be induced to secrete $Cl^-$ by addition of STa (Huott *et al*., 1988). Other intestinal cell lines like IEC-6 (rat cell line) can also bind STa; however, the receptor is not coupled to guanylate cyclase (Thompson and Gianella, 1990). Nevertheless, the relevance for humans of the data first obtained with rats was confirmed by studying the normal human intestinal mucosa (Cohen *et al*., 1986, 1988).

These studies revealed that STa receptors are found throughout the human intestine and colon with a decreasing number along the longitudinal axis of the gut (Krause *et al*., 1994). Specific binding of radioiodinated STa was noted in both crypts and villi of the small intestine and in crypts and surface epithelium of the colon (Field *et al*., 1989a, 1989b). Binding was also maximal in the villus preparations and decreased along the villus-to-crypt axis (Cohen *et al*., 1992).

A relationship was found between the number of STa receptors and age. The number was highest at birth, decreased in the first 1–3 weeks of life, and was stable thereafter (Cohen *et al*., 1988). The large number of receptors observed in infants could account for the severity of diarrhea due to STa in young children (Guarino *et al*., 1987c). A similar age-related difference in the number of STa receptors had already been observed in rats (Cohen *et al*., 1986).

A study by Swenson *et al*. (1996) indicated that GC-C was present in mouse intestinal crypts and villi of adult small intestine and surface epithelium of the colon. The presence of GC-C in mouse intestinal crypts supports the putative role of GC-C in fluid secretion and electrolyte homeostasis and resembles the pattern seen with human tissues.

## Alternative STa receptor

A number of smaller STa-binding proteins previously identified cross-reacts with anti-GC-C antibodies, indicating that these polypeptides could be the result of GC-C extracellular binding domain proteolysis (Cohen *et al*., 1993; Vaandrager *et al*., 1993a; Scheving and Chong, 1997). This observation can most probably account for numerous proteins with varying Mr previously described as STa receptors. In fact, similar lower

molecular mass STa-binding proteins were also observed in cells transfected to express GC-C, supporting the occurrence of processing and/or proteolysis of a single STa binding molecule (de Sauvage *et al*., 1992; Vaandrager *et al*., 1993a; Hirayama *et al*., 1992).

Now, at least one receptor for STa has been clearly identified as being GC-C. Nevertheless, the existence of other receptors for STa was suggested by different studies. Non GC-C-linked STa receptors may also exist and those could be linked to more than a single signal transduction system as will be discussed later.

## Mechanism of action

STa is a highly potent toxin with a rapid action (reaching maximal levels of cGMP within 5 min) but of short duration. For example, 6 ng of STa result in a positive fluid response in mouse intestine compared with 200 ng of STb or CT in the same model (Hitotsubashi *et al*., 1992a, 1992b). STa (and guanylin) binds to particulate guanylate cyclase in the jejunum and ileum brush border, leading to elevation of cGMP levels. GC-C is found primarily, but not exclusively, in intestinal cells lining the small intestine (Krause *et al*., 1994).

STa enterotoxin utilizes GC-C to alter ion transport in the gut. Following binding of STa to its receptor, a cascade of events ensues (Figure 48.3). The end result of STa action is the inhibition of $Na^+$-coupled-$Cl^-$ absorption in villus tips and stimulation of electrogenic $Cl^-$ secretion in crypt cells, resulting in a net fluid secretion in the lumen of the intestine (Forte *et al*., 1992; Dreyfus *et al*., 1984a; Huott *et al*., 1988; Field *et al*., 1978).

Inhibition of NaCl absorption could be attributed to binding of STa to enterocyte villi responsible for NaCl absorption (Almenoff *et al*., 1993; Cohen *et al*., 1992). Treatment of Caco-2 cells with STa prevented uptake of taurine, which is coupled to $Na^+$ absorption. This mechanism could also contribute to decrease the absorption of sodium (Brandsch *et al*., 1995).

GC-C is composed of a receptor, a transmembrane domain, a kinase-like domain, and a catalytic domain. In the basal state, GC-C is a homotrimer. After binding of a STa molecule, a bond between two of the three subunits is stabilized, as activation of GC-C may require the interaction of STa with two cyclase domains. Thus, internal dimerization within the homotrimeric GC-C appears as a key step in activation of guanylate cyclase by STa (Vaandrager *et al*., 1994). Rudner *et al*. (1995) recently proposed that the binding of STa to GC-C removes the inhibitory effects of the kinase-homology domain and promotes an interaction between the GC catalytic domains. Adenine nucleotides were shown to regulate the binding of STa to its receptor. It actually

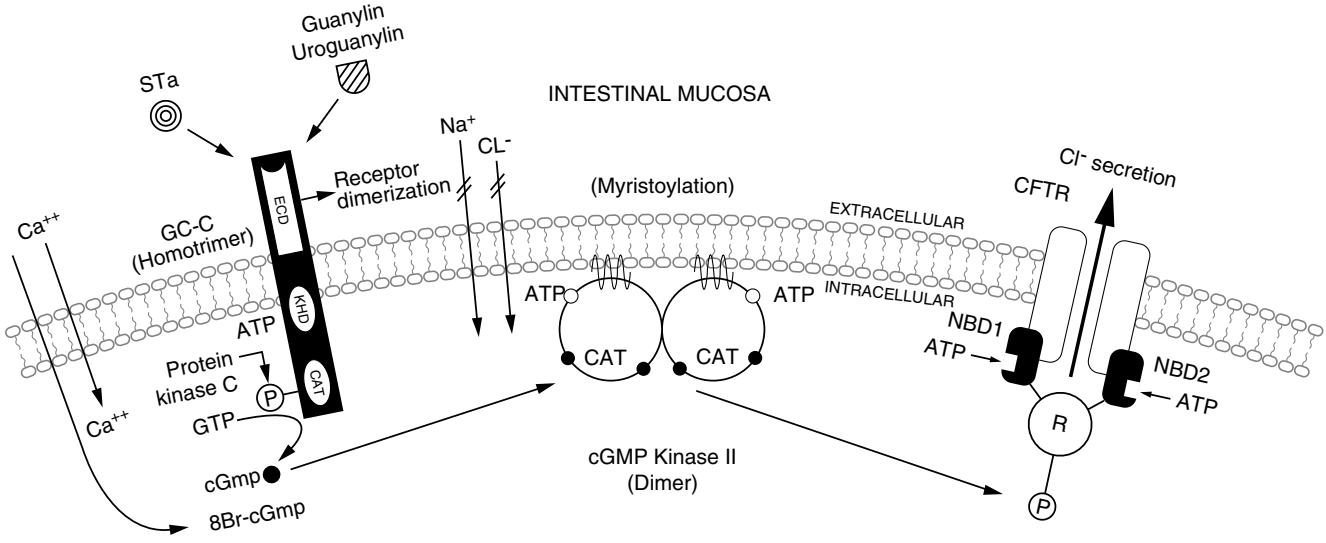



**FIGURE 48.3** Molecular model for the induction of a secretory response by STa toxin. STa and guanylin (uroguanylin) interact with guanylate cyclase C. GC-C is a homotrimer composed of a receptor domain (ECD), a transmembrane domain, a kinase-homology domain (KDH), and a catalytic domain (CAT). Interaction of STa with the receptor domain results in dimerization of two of the three CAT domains. Following STa-receptor interaction, $Ca^{++}$ influx occurs and this event activates protein kinase C, which phosphorylate CAT. Conversion of GTP in cGMP takes place and the $Na^+$ -$Cl^-$-coupled influx is blocked. cGMP or 8 br-cGMP but not 8-br-cAMP can then activate a cGMP-dependent protein kinase II (cGMP kinase II), which is a dimer molecule linked to the membrane via myristoyl molecules. The catalytic (CAT) domain of cGMP kinase II phosphorylates the R (Regulatory) domain of CFTR (cystic fibrosis transmembrane conductance regulator), a $Cl^-$ conductory pore. The R domain interacts with NDB1 and NDB2 (nucleotide binding domains). Then $Cl^-$ secretion is achieved via the CFTR channel. The cGMP-signaling enzyme (GC-C), the signal transducer cGMP kinase II, and the effector (CFTR) are co-localized on the membrane of intestinal epithelial cells.

decreases binding, but stimulates guanylate cyclase activity (Gazzano *et al*., 1991; Katwa *et al*., 1992). Activation of GC-C results in an increased level of cGMP, resulting in a net fluid secretion involving $Cl^-$.

An interaction between protein kinase C and the STa receptor was suggested by Weikel *et al*. (1990), who showed that treatment with phorbol ester, an active analogue of PKC, doubled STa-stimulated cGMP production in cultured intestinal cells. This increase was also observed by Crane *et al*. (1990) in STa-stimulated T84 cells. Crane *et al*. (1992) later demonstrated that treatment of T84 cells with purified PKC similarly increased STa-stimulated guanylyl cyclase activity. This increased response was blocked by PKC inhibitors and by ATP-δ-S, a biologically non-hydrolysable ATP analogue, indicating that PKC regulated the STa receptor through a phosphorylation step. They also presented evidence that PKC could phosphorylate and activate the STa receptor both *in vivo* and *in vitro*. In addition, using synthetic peptides, they have indicated that Ser-1029 of the STa receptor is most probably a phosphorylation site for PKC. A study by Wada *et al*. (1996) supported this hypothesis. It can thus be concluded that PKC activity in target cells modulates the cellular sensitivity to *E. coli* STa enterotoxin.

*In vivo*, the increase in cGMP results in a cGMP-dependent protein kinase activation of a linear chloride channel known as CFTR (cystic fibrosis transmembrane conductance regulator). CFTR is a $Cl^-$ channel encoded by the cystic fibrosis gene. The role of CFTR in secretion was confirmed by the observation of a lack of potential difference response, but not of a cGMP response to STa in the colon of cystic fibrosis (CF) patients (Goldstein *et al*., 1994). In addition, CFTR knockout mice were insensitive to STa (Pfeifer *et al*., 1996).

The CFTR protein contains two transmembrane domains forming the $Cl^-$ conducting pore, two nucleotide-binding domains, and a regulatory domain, where multiple phosphorylation sites for cAK (Gadsby and Nairm, 1994) and cGK (French *et al*., 1995), which are cAMP- and cGMP-dependent kinases, respectively, are present.

*In vivo*, following STa binding of STa to its receptor, only cyclic GMP-dependent kinase type II (cGKII) can effectively phosphorylate the R domain and gate the CFTR $Cl^-$ channel (French *et al*., 1995; Vaandrager *et al*., 1997a). cGKII is a membrane-associated cGMP-dependent kinase, whereas cGKI is a soluble kinase. cGMP cross-activation of cAK does not occur in native intestinal epithelium, but is observed *in vitro* in cGK-deficient T84 intestinal tumor cell lines (Forte *et al*., 1992; Lin *et al*., 1992; Tien *et al*., 1994). The level of activation and $Cl^-$ secretion of CFTR is dependent upon the level of phosphorylation (Akabes, 2000).

Recent studies strongly support a central role of cGKII and not cGKI or cAK as the signal transducer in STa- and cGMP-induced $Cl^-$ secretion (Vaandrager *et al*., 1997b). In fact, synthesis of endogenous cGKII in crypt cells corrolates well with the ability of STa and 8-Br-cGMP (and not 8-Br-cAMP) to stimulate $Cl^-$ secretion (Markert *et al*., 1995). STa could not stimulate $Cl^-$ secretion in the colon where cGKII is not found. Homozygous cGKII knockout mice and rat intestine pretreated with cGKII inhibitors lost the potential of intestinal $Cl^-$ secretion by STa and 8-Br-cGMP. A native membrane environment appears to be crucial for cGKII-selective phosphorylation of CFTR. The amino-terminal myristoyl groups present in cGKII dimer could be responsible for bringing cGKII and CFTR in close apposition so that the dimer can effectively phosphorylate and activate the channel, ultimately resulting in $Cl^-$ secretion (Vaandrager *et al*., 1996, 1997b).

The cGMP signaling enzyme (GC-C), the signal transducer (cGMP-dependent protein kinase II), and the effector (CFTR) are co-localized in the apical membrane of intestinal cells (Markert *et al*., 1995).

## STa action via alternative pathways

Non-GC-C STa receptor, if present, may be linked to alternative signaling pathways. Although the CFTR $Cl^-$ channel accounts for the main secretory pathway stimulated by STa, there is still a controversy as to whether cGMP is the sole signal transducer. In fact, as different pharmaceutical agents could alter the secretory response of STa, alternative pathways responsible for fluid secretion have been proposed (Table 48.1). Overall, to date, important major contradictory results have been reported from different research teams, adding to the perplexity of the situation. For example, as STa-secretory response was completely abolished by 5-hydroxytryptamine (5-HT or serotonin) receptor antagonists, this secretagogue has been proposed as an important mediator of secretion (Beubler *et al*., 1992). Interestingly, in these experiments, the cGMP response was not influenced. Thus, 5-HT could possibly mediate STa secretion by affecting prostaglandin synthesis (5HT-2 receptors) and/or neurons (5HT-3 receptors) (Lundgren, 2004). In support of this mechanism of action, it was shown that the cyclooxygenase inhibitor indomethacin caused an inhibition of STa-mediated fluid secretion in suckling mice (Madsen and Knoop, 1978). However, this inhibition was not observed by Kuhn *et al*. (1994) in the human intestine. Thus, the potential role of 5-HT and prostaglandin or leukotrienes remains controversial.

In fact, studies involving prostaglandin synthesis inhibitors and isolated rat intestinal epithelial cells

**TABLE 48.1** STa proposed alternative mechanisms of action

| | |
|---|---|
| 5-Hydroxytryptamine (serotonin) | Beubler *et al*. (1992) |
| | Madsen and Knoop (1978) |
| Prostaglandins/Leukotrienes | Greenberg *et al*. (1982) |
| | Hayden *et al*. (1996) |
| | Thomas and Knoop (1978) |
| | Greenberg *et al*. (1982) |
| Calcium/PKC | Thompson and Knoop (1982) |
| | Banik and Ganguly (1988, 1989) |
| | Chandhuri *et al*. (1993) |
| | Chandhuri and Ganguli (1995) |
| | Guanguly and Talukder (1985) |
| | Knoop *et al*. (1991) |
| | Chandhuri *et al*. (1993) |
| Neural activity | Eklund *et al*. (1985) |
| | Mathias *et al*. (1982) |
| | Rolfe and Levin (1994) |
| | Giannella *et al*. (1983) |
| | Roussel *et al*. (1992) |
| F-actin /C fibre | Matthews *et al*. (1993) |
| | Nzegwu and Levin (1996) |
| | Rolfe and Levin (1994) |

suggested that STa secretion could result from phosphatidylinositol and diacylglycerol release coupled with elevation of intracellular calcium levels and activation of protein kinase C (Greenberg *et al*., 1982; Thomas and Knoop, 1982; Banik and Ganguly, 1988, 1989; Chaudhuri *et al*., 1993; Chaudhuri and Ganguly, 1995; Ganguly and Talukder, 1985; Knoop *et al*., 1991).

In contrast, Dreyfus *et al*. (1984a) refuted the evidence for a role of prostaglandins and leukotrienes, calcium, and/or calmodulin in STa secretory action. Supporting this study, it was shown that STa increased cGMP levels in T84 cells, resulting in $Cl^-$ secretion, although no elevation of calcium levels and no hydrolysis of phospholipids were noted (Huott *et al*., 1988; Vajanaphanich *et al*., 1993).

STa was shown to cause changes in the myoelectric activity of the small intestine, indicating that the enteric nervous system could play a role in STa secretion. Following the use of neuronal inhibitors, a reduction in STa secretory response was observed *in vivo* and *in vitro* (Eklund *et al*., 1985; Mathias *et al*., 1982; Rolfe and Levin, 1994). The observed changes in myoelectric activity could result in the loss of normal peristaltism (Gianella *et al*., 1983; Roussel *et al*., 1992).

Finally, the secretory response of T84 cells to STa was reported to involve microfilament (F-actin) rearrangement at the basal pole of these polarized cells (Matthews *et al*., 1993). More recently, Rolfe and Levin (1994) showed that STa-activated electrogenic $Cl^-$ secretion through a myenteric secretory reflex with afferent C fiber component. A study by Nzegwu and Levin (1996) corroborated this observation.

For now, no definitive consensus can be reached with respect to the alternative pathway that STa could use to stimulate fluid secretion.

## *E. coli*-EAST1 TOXIN

### Generalities

Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) is a small protein, which was recognized more than a decade ago in an enteroaggregative *E. coli* (EAEC) strain isolated from the stools of a child suffering from diarrhea (Levine *et al*., 1988; Savarino *et al*., 1991). The EAST1-coding gene, *astA*, is not only found in EAEC, but also in other categories of *E. coli* responsible for diarrhea (Ménard and Dubreuil, 2002). Strains expressing EAST1 were shown to induce diarrhea principally in humans, but were also associated with disease in piglets and calves. EAST1 toxin has been proposed as a virulence factor implicated in the pathogenesis of EAEC, and it could play a role in the virulence of other enteropathogens as well (Paiva de Sousa and Dubreuil, 2001). This toxin is often compared to *E. coli* STa enterotoxin because it shares some physical and mechanistic similarities. However, it does not react with anti-STa antibodies or hybridize with DNA probes specific for STa (Savarino *et al*., 1991).

### Enteroaggregative *E. coli*

EAEC can adhere to HEp-2 cells and other cell surfaces in a characteristic aggregative pattern, sometimes described as a stacked-brick configuration. For many years, EAEC has been recognized as an agent of diarrhea, mostly in children living in developing countries (Nataro *et al*., 1993; Bhan *et al*., 1989). The adherence factors associated with EAEC are the aggregative adherence fimbriae I and II (Nataro *et al*., 1993; Savarino *et al*., 1994; Nataro *et al*., 1995). These fimbriae are morphologically and genetically different, but both are implicated in the aggregative adherence phenotype observed. Several other putative or established virulence factors are associated with EAEC, such as a 108-kDa cytotoxin named Pet (for plasmid-encoded toxin), hemolysins, various outer membrane proteins, and the enterotoxin designated EAST1 (Nataro and Kaper, 1998; Nataro *et al*., 1998; Law and Chart, 1998; Yamamoto *et al*., 1997b). However, not all studies indicate that EAEC is an agent of diarrhea (Nataro *et al*., 1998; Law and Chart, 1998). EAEC may represent an opportunistic pathogen for children or immunocompromised persons (Wanke *et al*., 1998). Moreover, virulence factors associated with EAEC strains are multiple, and deciphering their individual roles in disease is

complex. Among these virulence factors, EAST1 toxin seems implicated in the pathogenesis of EAEC leading to diarrhea. The role of EAST1 in diarrhea is still questioned because some volunteers challenged with EAST1-producing EAEC strains did not develop diarrhea even if the strains could effectively colonize the intestine (Nataro *et al*., 1995).

Some studies demonstrated that the presence of *E. coli astA*-positive in the stools of adults and children without diarrhea can be as high as 38% (Savarino *et al*., 1996; Gascon *et al*., 1998; Vila *et al*., 1998; Stephan and Untermann, 1999; Vila *et al*., 2000; Gascon *et al*., 2000). The existence of EAST1-producing *E. coli* in healthy subjects suggests that some persons could be asymptomatic carriers and that these strains could provoke diarrhea only under certain conditions.

The prototype EAEC strain 17-2 was isolated from a Chilean child with diarrhea (Levine *et al*., 1988). This strain caused diarrhea and sometimes death in a gnotobiotic piglet model (Tzipori *et al*., 1992). In pig, the moderate hyperemia of the distal small intestine and caecum, the swelling of small intestinal villi, plus the absence of an inflammatory response were pointing toward an enterotoxin-induced diarrhea (Tzipori *et al*., 1992).

## EAST1 in humans and animals

First associated with EAEC, subsequent studies demonstrated that EAST1 gene was also present in other diarrhea-causing *E. coli* and also in some other human enteric pathogens, such as *Salmonella* (Savarino *et al*., 1996; Paiva de Sousa and Dubreuil, 2001). EAST1 is not associated with a particular *E. coli* serotype, even though it may be more prevalent in some serogroups (Paiva de Sousa and Dubreuil, 2001; Rich *et al*., 1999; Yamamoto and Taneike, 2000). EAST1-producing *E. coli* have been primarily associated with diarrhea in children, but were later found in adults as well (Savarino *et al*., 1996; Vila *et al*., 1998). The prevalence of *astA* in EAEC strains isolated from stools from humans with diarrhea varies from 26–86.6% (Savarino *et al*., 1993, 1996; Paiva de Sousa and Dubreuil, 2001; Gascon *et al*., 1998; Yamamoto and Echeverria, 1996). Some *E. coli* strains isolated from humans with diarrhea were found to harbor no known virulence determinant other than EAST1 (Paiva de Sousa and Dubreuil, 2001; Nishikawa *et al*., 1999; Zhou *et al*., 2002). EAST1 is not only found in human strains but also in *E. coli* strains associated with diarrhea in cattle and pigs (Yamamoto and Echeverria, 1996; Yamamoto and Nakazawa, 1997; Choi *et al*., 2001a, 2001b; Bertin *et al*., 1998). For example, in a study on 476 *E. coli* strains isolated from weaned pigs with diarrhea and/or edema, 31.3% of the strains carried the EAST1 gene. Among them, 44.3% harbored no known fimbrial or other toxin gene (Choi *et al*., 2001b). Another study indicated that there may be a link between CS31A adhesin and EAST1 in pathogenic bovine *E. coli* (Bertin *et al*., 1998).

## Toxicity evaluation

EAST1 toxicity is generally detected in the Ussing chamber using rabbit intestinal tissues (Savarino *et al*., 1991). Enterotoxicity of culture filtrates of EAST1-producing ETEC strains has also been observed in the suckling mouse assay (McVeigh *et al*., 2000). ETEC strains harboring more than one copy of *astA* were toxic in the mouse assay, while bacteria with a single copy of the gene were not (McVeigh *et al*., 2000). It is tempting to hypothesize that detection of enteroactivity in either test may depend on the amount of EAST1 produced and present in the culture filtrates. Concordance between the presence of *astA* and toxicity of live bacterial strains or culture filtrates has been assayed and a correlation could be established for several strains (Savarino *et al*., 1996). However, the Ussing chamber assay can, for unknown reasons, fail to detect production of EAST1, as it was observed for EAEC strains provoking acute diarrhea in Brazilian children (Savarino *et al*., 1996; Monteiro-Neto *et al*., 1997). This result could be explained by a lack of toxin expression under *in vitro* growth conditions or due to a mutation silencing the EAST1 gene without altering significantly its nucleotide sequence (Savarino *et al*., 1996).

## EAST1 polypeptide

The amino acid sequence of EAST1 was deduced from the open reading frame named *astA*. EAST1 is a 38 amino acid peptide with a molecular weight of 4100 Da and a calculated pI of 9.25 (Figure 48.4)(Savarino *et al*., 1993). Knowledge on EAST1 was acquired through the use of culture filtrates or use of EAST1-producing strains, as EAST1 toxin was only recently purified (Ménard *et al*., 2004). Fractions of culture filtrates of EAEC strain 17-2, heated at 65°C for 15 min, demonstrated partial heat stability (Savarino *et al*., 1991). Among the 38 amino acids composing EAST1, there are four cysteines at positions 17, 20, 24, and 27, which were proposed to be involved in the formation of two disulfide bridges. Indirect evidence for the importance of disulfide bridges' integrity for toxicity expression was determined when cysteine 17 was replaced by an alanine abolishing the toxicity (Fasano, 1999; Uzzau and Fasano, 2000). Unlike STa and STb, a classic signal peptide was not observed in the $NH_2$-terminus of the predicted EAST1 sequence (Savarino *et al*., 1993). Polyclonal anti-STa antibody could not neutralize the

biological activity of EAST1 in Ussing chamber. Thus, EAST1 was defined as being immunologically different from STa (Savarino *et al*., 1991). A synthetic peptide composed of residues 8 to 29 of EAST1 induced a response in Ussing chamber, indicating that the toxic activity was contained within this large fragment (Savarino *et al*., 1993). Because EAST1 bears some similarities to STa, the suckling mouse assay, the standard bioassay for STa, was proposed to evaluate enterotoxicity. Using concentrates (20X and 50X) of culture supernatant (fraction between 2 and 5 kDa) of strain 17-2 and six other EAEC strains EAST1-positive isolates produced a gut-to-body-weight ratio, in CD-1 mouse strain, below the determined threshold for the bioassay (Savarino *et al*., 1991). It was concluded that EAST1 toxin could not be detected by the suckling mouse assay under conditions in which STa was active.

### *AstA gene*

The enterotoxin EAST1 is encoded by the gene *astA*, a 117-bp-long DNA sequence. There is no homology between *astA* and *estA*, the structural gene for STa. The G+C content of *astA* is 53%, which is similar to the mean value for *E. coli* (50.8%), while for *estA* (coding for STa) it is 30.6% (Savarino *et al*., 1993; Lai *et al*., 1999). *astA* has an atypically low codon adaptation index (0.132), which represents a deviation from the preferred codon usage for *E. coli* (McVeigh *et al*., 2000). The presence of *astA* in EAEC strains was associated with a 60-MDa plasmid mediating aggregative adherence, but was also observed on other plasmids, in EAEC, and other bacteria (Savarino *et al*., 1993; Savarino *et al*., 1996; Yamamoto *et al*., 1996). EAST1 gene is strongly linked to the aggregative adherence fimbriae I (AAF/I), implicated in the aggregative adherence phenotype. Both EAST1 and AAF/I genes are on a 60-MDa plasmid found in EAEC strain 17-2 (Nataro *et al*., 1993). Czeczulin *et al*. (1999) have shown that aggregative adherence plasmids are transmissible horizontally, presumably through conjugation. EAEC has a pattern of plasmid transmission similar to *Rhizobium* species, in which a virulence plasmid is acquired and then maintained among clonal descendants (Czeczulin *et al*., 1999). *astA* was observed on plasmids, but was also found on the chromosome of various bacteria in one or more copies (McVeigh *et al*., 2000). The presence of more than one copy of *astA* in bacteria was not restricted to ETEC, as one EHEC O157:H7 strain had two copies of *astA* (Savarino *et al*., 1996). The nucleotide sequence flanking the *astA* ORF has not been characterized yet, and no promoter or regulator of expression has been identified. Actually, very little is known about the genetic regulation of EAST1 expression. Nucleotide sequences upstream of *astA* from porcine and bovine ETEC strains were identical, but were divergent from human ETEC, DAEC, and EAEC (Yamamoto *et al*., 1996; Yamamoto and Echeverria, 1996; Yamamoto *et al*., 1997a, 1997b). There seems to be a certain consensus among flanking sequences of *astA*, even though the results obtained by Yamamoto and Nakazawa (1997) point toward a heterogeneity of DNA sequences between *E. coli* affecting humans and animals. As mentioned before, the EAST1 gene seems to be present among numerous diarrheagenic *E. coli* and also some *Salmonella agona* strains (Paiva de Sousa and Dubreuil, 2001). Several studies reported the presence of *astA* on transposon-like sequences, near insertion elements or inverted repeats, which could represent means of transmission for the EAST1 gene.

## Mechanism of action

The secondary messenger involved in the response of EAST1 was investigated among the following three molecules: cAMP, cGMP, and calcium. Only pretreament of tissue with 8-BrcGMP (a cGMP analogue) abrogated the biological response to a culture supernatant fraction (2–5 kDa) of strain 17-2, pointing toward cGMP as a possible second messenger involved in the mechanism of action of EAST1. Savarino *et al*. (1991) also observed that under both $Cl^-$-free and $HCO3^-$-free conditions, the short circuit current, for the 2- to 5-kDa fraction, was significantly less, suggesting that these ions may be involved in the generated electrical response. The small size and relative heat stability of this protein and the finding that cGMP may be acting as a second messenger led to the comparison of EAST1 with *E. coli* STa toxin.

In Ussing chamber, EAST1, STa, and STb induce a reaction in approximately 30 to 40 min (Fasano, 1999;

| Molecules | Number of residues | Mature amino acid sequences | References |
|---|---|---|---|
| **EAST1 17-2** | 38 | MPSTQYIRRPASSYASCIWC**T**TACASCHGRTTKPSLAT | Savarino *et al.* (1993) |
| **EAST1 0-42** | 38 | MPSTQYIRRPASSYASCIWC**A**TACASCHGRTTKPSLAT | Yamamoto *et al.* (1997) |

**FIGURE 48.4** Primary sequence of *Escherichia coli* EAST1 17-2 and O-42. In bold, the amino acid at position 21 that differs between these two variants. The box shows the partially conserved N-P-A-C sequence.

Thompson and Giannella, 1985; Dreyfus *et al*., 1983; Field *et al*., 1978). EAST1 protein shares 50% identity with the enterotoxic domain of STa (amino acid residues 6 to 18) (Savarino *et al*., 1993). By aligning STa family members (STaH and STaP) with guanylin and EAST1, the following common motif can be observed: a small nonpolar amino acid residue-alanine-cysteine-small nonpolar residue-small polar residue-cysteine (Figure 48.1) (Savarino *et al*., 1993; Uzzau and Fasano, 2000). EAST1 is structurally and functionally similar to guanylin, both having four cysteines and activating the production of cGMP (Savarino *et al*., 1991). A study on T84 cells and COS cells transfected with GC-C suggest that EAST1 interacts with GC-C to elicit an increase in cGMP (Fasano, 1999; Uzzau and Fasano, 2000). This interaction could occur through the N-P-A-C motif common to the ST toxins and partially conserved for EAST1 (i.e. X-X-A-C) (Figure 48.1).

### EAST1 variants

The first detected *astA* gene was in EAEC strain 17-2 and a variant of this gene was later observed in EAEC strain O-42 (Nataro *et al*., 1995; Yamamoto *et al*., 1997b). EAEC strain 17-2 (serotype O3:H2) was isolated from a Chilean child with diarrhea, while EAEC strain O-42 (O44:H18) was isolated from a Peruvian child with diarrhea (Savarino *et al*., 1991; Nataro *et al*., 1995; Henderson *et al*., 1999). This EAST1 variant differs from strain 17-2 sequence by one base at codon 21 (ACA→GCA), resulting in a change in the amino acid threonine to alanine. Nataro *et al*. (1995) observed heterogeneity in the virulence of EAEC strains 17-2 and O-42. As strain O-42 (sometimes referred to as strain JPN10) was able to provoke diarrhea in volunteers, whereas strain 17-2 did not, it has been proposed that the EAST1 variant of O-42 could contribute to the virulence of EAEC in a more significant way (Nataro *et al*., 1995; Yamamoto *et al*., 1997b). Moreover, the EAST1 toxin produced by strain O-42 can be observed more frequently in epidemiological studies than EAST1 of strain 17-2 (Yamamoto *et al*., 1997b; Lai *et al*., 1999). It should be specified that these two EAEC strains differ not only in their EAST1 sequence, but also in the fimbrial antigen they express. Strain 17-2 harbors AAF/I fimbrial antigen, while strain O-42 expresses AAF/II (Elias *et al*., 1999a and b; Nataro *et al*., 1996). Strains 17-2 and O-42 have distinct nucleotide sequences flanking *astA*. These differences may explain the variation of pathogenicity observed for those two variants (Nataro *et al*., 1995; Yamamoto *et al*., 1997b).

Other variants of EAST1 have been observed through various epidemiological studies (Yamamoto *et al*., 1997a, 1997b; Yamamoto and Taneike, 2000). These variants have usually been reported only once and probably represent less frequently distributed molecules. The toxicity of these occasionally observed variants has not been evaluated, thus precluding their importance as a virulence factor.

## *VIBRIO* AND *YERSINIA* STs

### Genetics

Significant divergence was noted between *E. coli* STaH and STaP and the NAG-ST gene (*stn*) of *V. cholerae* non-O1, although the three toxins share a similar DNA sequence at the COOH-terminal region. The gene *stn* is chromosomally encoded and despite the nucleotide sequence divergence with STa, the amino acid sequence shows considerable structural similarity, with respectively 39 or 36 of the 78 amino acids of NAG-ST being identical to STaH or STaP (Ogawa *et al*., 1990). The hydropathy plot analysis for the precursors, as well as the sequence identity analysis, suggested that NAG-ST and *E. coli* STa have a common evolutionary origin (Ogawa *et al*., 1990). *V. cholerae* O1-ST gene (*sto*) is very similar to *stn* and both genes are flanked by 123-bp direct repeats, and they both include inverted repeats with at least 93% homology (Ogawa and Takeda, 1993). Strains of *V. cholerae*, *V. mimicus*, *V. metschnikovii*, *V. hollisae*, and *Y. enterolitica* possess the direct repeat sequences, regardless of the presence of ST gene. The direct repeats appear to play a role in the evolutionary process by mediating chromosomal rearrangements. Nevertheless, not all *V. cholerae* have direct repeats sequences, indicating it could represent a transposable element or that it is unstable in the chromosome. Three transitions and three transversions between *sto* and *stn* ORFs are responsible for four codon changes without altering the mature ST amino acid sequences (Ogawa and Takeda, 1993). For *sto* and *stn*, in contrast to *estA*, no typical transposon or insertion sequence is found in the flanking region (Ogawa and Takeda, 1993).

Among *Y. enterolitica*, three defined molecular ST types are documented. They include Y-STa, Y-STb, and Y-STc (Yoshino *et al*., 1993, 1994). The structural gene for Y-STa (*ystA*) is located on the chromosome and encodes a polypeptide of 71 amino acids (Delor *et al*., 1990). Sequence comparisons between *ystA* and *estA1* (encoding STaH) indicate a divergence of 58% with only 23 conserved amino acids. At the 3′ end of the *ystA* gene it shows 80% homology, resulting in 11 out of 13 identical amino acids. Deduced precursor protein amino acid sequences of Y-STb showed more homology with Yk-ST (*Y. kristensii*) (68%) than with Y-STa (57%) and Y-STc (60%) (Ramamurthy *et al*., 1997).

## Polypeptide structure and biological property comparison

STs produced by diverse enteropathogenic bacteria share a highly conserved sequence of 13 amino acids residing in the COOH-terminal domain. This sequence includes six cysteines that are linked by three disulfide bonds with the position being conserved in all STs (Shimonishi *et al*., 1987; Hidaka *et al*., 1988). Peptides with only one disulfide bond show no biological activity. The shared sequence is the following: C-C-[E/D]-[L/I/V]-C-C-N-P-A-C-[A/T/F]-G-C (Nair and Takeda, 1998) (Figure 48.1). This tridecapeptide constitutes the minimal structure essential for toxicity. It has been designated as the "toxic domain" (Yoshimura *et al*., 1985). A four amino acid sequence (N-P-A-C) is present and conserved in all STs family members (Figure 48.1). This sequence was shown for STa and guanylin, the endogeneous ligand for GC-C, to be responsible for receptor binding (Greenberg *et al*., 1997).

The biological activity of STs is a function of the correct formation of the three disulfide bonds. These bridges are necessary for toxicity as they stabilize the spatial structure as was first shown for *E. coli* STaH (Yamasaki *et al*., 1988).

For *V. cholerae* O1-ST, the toxicity of ST is increased with the loss of the $NH_2$-terminal amino acids (Yoshino *et al*., 1983). A ST molecule truncated of the first 11 amino acids (O1-ST1, comprising 17 amino acids) has a minimal effective dose 10 times less than the entire toxin (28 amino acids)(O1-ST4) (Yoshino *et al*., 1993). STs produced by members of the genus *Vibrio* (O1-ST, NAG-ST, HT-ST, and M-ST) are less potent than *E. coli* STaH and *Y. enterolitica* Y-STb (Takao *et al*., 1985b; Arita *et al*., 1991 a,b; Yoshino *et al*., 1993).

Chimera-peptide analysis indicated that the higher potency of *Y. enterolitica* Y-STb over Y-STa (20 times more potent) is due to the presence of a -E instead of -N in position 20. The higher toxicity of Y-STb compared to Y-STa is also due to the substitution of -Y for -N in position 17 (Yoshino *et al*., 1994). Y-STc, a molecule comprising 53 amino acid residues and being the largest known ST, showed maximal potency as it has a lower minimal effective dose (4 times more potent) than *E. coli* STaH, which for many years was thought to represent the most toxic ST molecule. The COOH-terminal core sequence conserved domain is responsible for the heat stablility of STs, whereas the NH2-terminal region is not involved (Yoshino *et al*., 1995). In Y-STc, replacement of -N by -W contributes to higher potency of Y-STc compared to Y-STa (80 times more potent), and it also confers resistance to proteases (Aimoto, *et al*., 1983; Ikemura *et al*., 1984). *In vivo*, the COOH-terminal portion of Y-STc is susceptible to various proteases in the host's intestine.

## Maturation and secretion

Like *E. coli* STa toxin, ST precursor proteins comprise three regions, namely the pre-region, the pro-region, and the mature toxin. *V. cholerae* O1-ST is synthesized as a pre-pro-toxin (Ogawa and Takeda, 1993). Similar to other ST-producing bacteria, the signal sequence consists of 18 amino acids, and it is cleaved upon export to the periplasm (Sjostrom *et al*., 1987).

Y-STs are also produced as pre-pro-toxin (Okamoto *et al*., 1990; Rasheed *et al*., 1990). Y-STa is a 71 amino acid pre-pro-toxin that is processed like STa, but leaving a 30 amino acid mature toxin (Delor *et al*., 1990). A 53 amino acid peptide results from signal peptidase 1 cleavage. This pro-toxin is then translocated to the periplasm where the three essential disulfide bonds are formed by DsbA protein before secretion is possible (Yamanaka *et al*., 1994). The pro-region plays an important role in the formation of disulfide bonds, but it does not contribute to toxicity of the molecule. Since for Y-STc the pro-region consisting of 53 amino acids is secreted as the mature toxin, the Y-STc precursor is a useful tool in understanding the maturation and secretion processes of the ST toxin family (Yoshino *et al*., 1995; Huang *et al*., 1997). In fact, it implied that the proteolytic processing of a pro-toxin in the periplasm is not essential for the secretion of the mature toxin through the outer membrane (Rasheed *et al*., 1990; Yang *et al*., 1992). The existence of ST and its subtypes among *V. cholerae* and *Y. enterolitica* indicates that multiple enzymes are involved in the maturation process.

## Receptor

The STs act by binding to a receptor found on the brush border membrane of epithelial cells. A region sharing striking identity with STaH and STaP (amino acids 5–17 and 6–18, respectively) and ST of *V. cholerae* and *Y. enterolitica* is found (Figure 48.1). This region is known to confer binding and full enterotoxic activity. Despite this fact, for now, we can only speculate on the receptor involved and the mechanism of action retained by STs for toxicity expression.

Purified Y-ST stimulates increased cGMP levels in mouse intestine and in cells in culture (Inoue *et al*., 1983). Activation of GC-C results in increased levels of intracellular cGMP that stimulate chloride secretion and/or inhibit its adsorption, resulting in net intestinal fluid secretion. Although the toxic domain of STs

secreted by enteric bacteria including *V. cholerae* and *Y. enterolitica* shares a striking identity with *E. coli* STa, it is not proven that these STs elevate intracellular cyclic GMP via activation in a similar way to guanylate cyclase type C (Sears and Kaper, 1996). The N-P-A-C sequence known, for STa and guanylin, to bind to GC-C is found more or less conserved in all ST sequences (Figure 48.1). Nevertheless, the mechanism by which *Vibrio* and *Yersinia* STs stimulate intestinal secretion is thought to be similar or identical to that used by *E. coli* STa.

### Immogenicity and antigenic relationships

Due to their peptide nature, STs are poor immunogens. Neutralization assays using monoclonal antibodies specific for *E. coli* STaH and STaP and NAG-ST permitted the analysis of antigenic determinants of STs. These assays indicated that toxicity of Y-STa was abolished by NAG-ST monoclonal, but only weakly neutralized by *E. coli* STaH (Takeda *et al*., 1990). Y-STb toxicity was neutralized by both monoclonals. Y-STc activity was abolished by *E. coli* STaH monoclonal, but not by NAG-ST antibodies. Overall, this indicated that Y-STb and Y-STc appear more immunologically related to *E. coli* STaH than Y-STa.

## CONCLUSION

Almost 20 years of research have been devoted to the study of STa, positioning it as a prototype for the later discovered heat-stable toxins. These studies have led to the precise knowledge of the structure, toxic domain, and more recently the identification of the STa receptor. This information culminated rapidly in a general understanding of the secretory pathway exploited by STa. Now that CG-C has been identified as the STa receptor, cGKII as the signal transducer, and CFTR as the secretory effector, drugs that interfere either with the processes leading to secretion or directly on the $Cl^-$ channel could be used to abolish or limit STa action. Future studies should now be directed at determining, for STa, if the alternative secretory pathways are truly exploited and, if so, to what extent compared to the CFTR $Cl^-$ channel.

For now, even though ST toxins are produced by various bacterial groups, the links between the presence of ST gene, ST production, and the occurrence of diarrheal disease is not always clearly intertwined. Concerning *E. coli* EAST1 and STs produced by *Vibrio* and *Yersinia*, the identity of the receptor, as well as the mechanism of action by which they produce diarrhea, has to be demonstrated.

## REFERENCES


Aimoto, S., Watanabe, H., Ikemura, H., Shimonishi, Y., Takeda, T., Takeda, Y. and Miwatani, T. (1983). Chemical synthesis of a highly potent and heat-stable analog of an enterotoxin produced by a human strain of enterotoxigenic *Escherichia coli*. *Biochem. Biophys. Res. Commun*. **112,** 320–326.

Aitken, R. and Hirst, T.R. (1993). Recombinant enterotoxins as vaccines against *Escherichia coli*-mediated diarrhea. *Vaccine* **11,** 227–233.

Akabas, M.H. (2000). Cystic fibrosis transmembrane conductance regulator. Structure and function of an epithelial chloride channel. *J. Biol. Chem*. **275,** 3729–3732.

Alderete, J.F. and Robertson, D.C. (1977a). Nutrition and enterotoxin synthesis by enterotoxigenic strains of *Escherichia coli*: defined medium for production of heat-stable enterotoxin. *Infect. Immun*. **15,** 781–788.

Alderete, J.F. and Robertson, D.C. (1977b). Repression of heat-stable enterotoxin synthesis in enterotoxigenic *Escherichia coli*. *Infect. Immun*. **17,** 629–633.

Almenoff, J.S., Williams, S.I., Scheving, L.A., Judd, A.K. and Schoolnik, G.K. (1993). Ligand-based histochemical localization and capture of cells expressing heat-stable enterotoxin receptors. *Mol. Microbiol*. **8,** 865–873.

Amirmozafari, N. and Robertson, D.C. (1993). Nutritional requirements for synthesis of heat-stable enterotoxin by *Yersinia enterocolitica*. *Appl. Environ. Microbiol*. **59,** 3314–3320.

Arita, M., Honda, T., Miwatani, T., Ohmori, K., Takao, T. and Shimonishi, Y. (1991a). Purification and characterization of a new heat-stable enterotoxin produced by *Vibrio cholerae* non-O1 serogroup Hakata. *Infect. Immun*. **59,** 2186–2188.

Arita, M., Honda, T., Miwatani, T., Takeda, T., Takao, T. and Shimonishi, Y. (1991b). Purification and characterization of a heat-stable enterotoxin of *Vibrio mimicus*. *FEMS Microbiol. Lett*. **63,** 105–110.

Arita, M., Takeda, T., Honda, T. and Miwatani, T. (1986). Purification and characterization of *Vibrio cholerae* non-O1 heat-stable enterotoxin. *Infect. Immun*. **52,** 45–49.

Banik, N. and Ganguly, U. (1988). Stimulation of phosphoinositides breakdown by the heat stable *E. coli* enterotoxin in rat intestinal epithelial cells. *FEBS Lett*. **236,** 489–492.

Banik, N.D. and Ganguly, U. (1989). Diacylglycerol breakdown in plasma membrane of rat intestinal epithelial cells. Effect of *E. coli* heat-stable toxin. *FEBS Lett*. **250,** 201–204.

Bertin, Y., Martin, C., Girardeau, J.P., Pohl, P. and Contrepois, M. (1998). Association of genes encoding P fimbriae, CS31A antigen, and EAST 1 toxin among CNF1-producing *Escherichia coli* strains from cattle with septicemia and diarrhea. *FEMS Microbiol. Lett*. **162,** 235–239.

Beubler, E., Badhri, P. and Schirgi-Degen, A. (1992). 5-HT receptor antagonists and heat-stable *Escherichia coli* enterotoxin-induced effects in the rat. *Eur. J. Pharmacol*. **219,** 445–450.

Bhan, M.K., Khoshoo, V., Sommerfelt, H., Raj, P., Sazawal, S. and Srivastava, R. (1989). Enteroaggregative *Escherichia coli* and *Salmonella* associated with nondysenteric persistent diarrhea. *Pediatr. Infect. Dis. J*. **8,** 499–502.

Boyce, J.M., Evans, E.J., Jr., Evans, D.G. and DuPont, H.L. (1979). Production of heat-stable, methanol-soluble enterotoxin by *Yersinia enterocolitica*. *Infect. Immun*. **25,** 532–537.

Brandsch, M., Ramamoorthy, S., Marczin, N., Catravas, J.D., Leibach, J.W., Ganapathy, V. and Leibach, F.H. (1995). Regulation of taurine transport by *Escherichia coli* heat-stable enterotoxin and guanylin in human intestinal cell lines. *J. Clin. Invest*. **96,** 361–369.

Brandwein, H., Deutsch, A., Thompson, M. and Giannella, R. (1985). Production of neutralizing monoclonal antibodies to *Escherichia coli* heat-stable enterotoxin. *Infect. Immun*. **47,** 242–246.

Burgess, M.N., Bywater, R.J., Cowley, C.M., Mullan, N.A. and Newsome, P.M. (1978). Biological evaluation of a methanol-soluble, heat-stable *Escherichia coli* enterotoxin in infant mice, pigs, rabbits, and calves. *Infect. Immun*. **21,** 526–531.

Carpick, B.W. and Gariepy, J. (1991). Structural characterization of functionally important regions of the *Escherichia coli* heat-stable enterotoxin STIb. *Biochemistry* **30,** 4803–4809.

Carpick, B.W. and Gariepy, J. (1993). The *Escherichia coli* heat-stable enterotoxin is a long-lived superagonist of guanylin. *Infect. Immun*. **61,** 4710–4715.

Chang, M.S., Lowe, D.G., Lewis, M., Hellmiss, R., Chen, E. and Goeddel, D.V. (1989). Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* **341,** 68–72.

Chaudhuri, A.G. and Ganguly, U. (1995). Evidence for stimulation of the inositol triphosphate-Ca2+ signaling system in rat enterocytes by heat-stable enterotoxin of *Escherichia coli. Biochim. Biophys. Acta* **1267,** 131–133.

Chaudhuri, A.G., Sen, P.C. and Ganguly, U. (1993). Evidence for protein kinase C stimulation in rat enterocytes pretreated with heat-stable enterotoxin of *Escherichia coli. FEMS Microbiol. Lett*. **110,** 185–189.

Choi, C., Cho, W., Chung, H., Jung, T., Kim, J. and Chae, C. (2001a). Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1) gene in isolates in weaned pigs with diarrhea and/or edema disease. *Vet. Microbiol*. **81,** 65–71.

Choi, C., Kwon, D. and Chae, C. (2001b). Prevalence of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene and its relationship with fimbrial and enterotoxin genes in *E. coli* isolated from diarrheic piglets. *J. Vet. Diagn. Invest*. **13,** 26–29.

Cohen, M.B., Guarino, A., Shukla, R. and Giannella, R.A. (1988). Age-related differences in receptors for *Escherichia coli* heat-stable enterotoxin in the small and large intestine of children. *Gastroenterology* **94,** 367–373.

Cohen, M.B., Jensen, N.J., Hawkins, J.A., Mann, E.A., Thompson, M.R., Lentze, M.J. and Giannella, R.A. (1993). Receptors for *Escherichia coli* heat-stable enterotoxin in human intestine and in a human intestinal cell line (Caco-2). *J. Cell Physiol*. **156,** 138–144.

Cohen, M.B., Mann, E.A., Lau, C., Henning, S.J. and Giannella, R.A. (1992). A gradient in expression of the *Escherichia coli* heat-stable enterotoxin receptor exists along the villus-to-crypt axis of rat small intestine. *Biochem. Biophys. Res. Commun*. **186,** 483–490.

Cohen, M.B., Moyer, M.S., Luttrell, M. and Giannella, R.A. (1986). The immature rat small intestine exhibits an increased sensitivity and response to *Escherichia coli* heat-stable enterotoxin. *Pediatr. Res*. **20,** 555–560.

Cover, T.L. and Aber, R.C. (1989). *Yersinia enterocolitica. N. Engl. J. Med*. **321,** 16–24.

Crane, J.K., Burrell, L.L., Weikel, C.S. and Guerrant, R.L. (1990). Carbachol mimics phorbol esters in its ability to enhance cyclic GMP production by STa, the heat-stable toxin of *Escherichia coli. FEBS Lett*. **274,** 199–202.

Crane, J.K., Wehner, M.S., Bolen, E.J., Sando, J.J., Linden, J., Guerrant, R.L. and Sears, C.L. (1992). Regulation of intestinal guanylate cyclase by the heat-stable enterotoxin of *Escherichia coli* (STa) and protein kinase C. *Infect. Immun*. **60,** 5004–5012.

Currie, M.G., Fok, K.F., Kato, J., Moore, R.J., Hamra, F.K., Duffin, K.L. and Smith, C.E. (1992). Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc. Natl. Acad. Sci. USA* **89,** 947–951.

Czeczulin, J.R., Whittam, T.S., Henderson, I.R., Navarro-Garcia, F. and Nataro, J.P. (1999). Phylogenetic analysis of enteroaggregative and diffusely adherent *Escherichia coli. Infect. Immun*. **67,** 2692–2699.

de Sauvage, F.J., Camerato, T.R. and Goeddel, D.V. (1991). Primary structure and functional expression of the human receptor for *Escherichia coli* heat-stable enterotoxin. *J. Biol. Chem*. **266,** 17912–17918.

de Sauvage, F.J., Horuk, R., Bennett, G., Quan, C., Burnier, J.P. and Goeddel, D.V. (1992). Characterization of the recombinant human receptor for *Escherichia coli* heat-stable enterotoxin. *J. Biol. Chem*. **267,** 6479–6482.

Delor, I., Kaeckenbeeck, A., Wauters, G. and Cornelis, G.R. (1990). Nucleotide sequence of yst, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and non-pathogenic *Yersiniae. Infect. Immun*. **58,** 2983–2988.

Dreyfus, L.A., Frantz, J.C. and Robertson, D.C. (1983). Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. *Infect. Immun*. **42,** 539–548.

Dreyfus, L.A., Jaso-Friedmann, L. and Robertson, D.C. (1984a). Characterization of the mechanism of action of *Escherichia coli* heat-stable enterotoxin. *Infect. Immun*. **44,** 493–501.

Dreyfus, L.A. and Robertson, D.C. (1984b). Solubilization and partial characterization of the intestinal receptor for *Escherichia coli* heat-stable enterotoxin. *Infect. Immun*. **46,** 537–543.

Echeverria, P., Verhaert, L., Ulyangco, C.V., Komalarini, S., Ho, M.T., Orskov, F. and Orskov, I. (1978). Antimicrobial resistance and enterotoxin production among isolates of *Escherichia coli* in the Far East. *Lancet* **2,** 589–592.

Eklund, S., Jodal, M. and Lundgren, O. (1985). The enteric nervous system participates in the secretory response to the heat-stable enterotoxins of *Escherichia coli* in rats and cats. *Neuroscience* **14,** 673–681.

Elias, W.P., Jr., Czeczulin, J.R., Henderson, I.R., Trabulsi, L.R. and Nataro, J.P. (1999a). Organization of biogenesis genes for aggregative adherence fimbria II defines a virulence gene cluster in enteroaggregative *Escherichia coli. J. Bacteriol*. **181,** 1779–1785.

Elias, W.P., Suzart, S., Trabulsi, L.R., Nataro, J.P. and Gomes, T.A. (1999b). Distribution of aggA and aafA gene sequences among *Escherichia coli* isolates with genotypic or phenotypic characteristics, or both, of enteroaggregative *E. coli. J. Med. Microbiol*. **48,** 597–599.

Fasano, A. (1999). Cellular microbiology: can we learn cell physiology from microorganisms? *Am. J. Physiol*. **276,** C765–776.

Field, M., Graf, L.H., Jr., Laird, W.J. and Smith, P.L. (1978). Heat-stable enterotoxin of *Escherichia coli*: *in vitro* effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proc. Natl. Acad. Sci. USA* **75,** 2800–2804.

Field, M., Rao, M.C. and Chang, E.B. (1989a). Intestinal electrolyte transport and diarrheal disease (1). *N. Engl. J. Med*. **321,** 800–806.

Field, M., Rao, M.C. and Chang, E.B. (1989b). Intestinal electrolyte transport and diarrheal disease (2). *N. Engl. J. Med*. **321,** 879–883.

Forte, L.R., Eber, S.L., Turner, J.T., Freeman, R.H., Fok, K.F. and Currie, M.G. (1993). Guanylin stimulation of $Cl^-$ secretion in human intestinal T84 cells via cyclic guanosine monophosphate. *J. Clin. Invest*. **91,** 2423–2428.

Forte, L.R., Thorne, P.K., Eber, S.L., Krause, W.J., Freeman, R.H., Francis, S.H. and Corbin, J.D. (1992). Stimulation of intestinal $Cl^-$ transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol*. **263,** C607–615.

French, P.J., Bijman, J., Edixhoven, M., Vaandrager, A.B., Scholte, B.J., Lohmann, S.M., Nairn, A.C. and de Jonge, H.R. (1995). Isotype-specific activation of cystic fibrosis transmembrane conductance regulator-chloride channels by cGMP-dependent protein kinase II. *J. Biol. Chem*. **270,** 26626–26631.

Gadsby, D.C. and Nairn, A.C. (1994). Regulation of CFTR channel gating. *Trends Biochem. Sci*. **19,** 513–518.

Ganguly, U. and Talukder, S. (1985). Effect of *Escherichia coli* heat-stable enterotoxin (ST) on calcium uptake by rat intestinal brush border membrane vesicles (BBMV). *FEMS Microbiol. Lett*. **26,** 255–257.

Garbers, D.L. (1991). The guanylyl cyclase-receptor family. *Can. J. Physiol. Pharmacol*. **69,** 1618–1621.

Gariepy, J., Judd, A.K. and Schoolnik, G.K. (1987). Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin ST1b. *Proc. Natl. Acad. Sci. USA* **84,** 8907–8911.

Gariepy, J., Lane, A., Frayman, F., Wilbur, D., Robien, W., Schoolnik, G.K. and Jardetzky, O. (1986). Structure of the toxic domain of the *Escherichia coli* heat-stable enterotoxin ST I. *Biochemistry* **25,** 7854–7866.

Gascon, J., Vargas, M., Quinto, L., Corachan, M., Jimenez de Anta, M.T. and Vila, J. (1998). Enteroaggregative *Escherichia coli* strains as a cause of traveler's diarrhea: a case-control study. *J. Infect. Dis*. **177,** 1409–1412.

Gascon, J., Vargas, M., Schellenberg, D., Urassa, H., Casals, C., Kahigwa, E., Aponte, J.J., Mshinda, H. and Vila, J. (2000). Diarrhea in children under 5 years of age from Ifakara, Tanzania: a case-control study. *J. Clin. Microbiol*. **38,** 4459–4462.

Gazzano, H., Wu, H.I. and Waldman, S.A. (1991). Activation of particulate guanylate cyclase by *Escherichia coli* heat-stable enterotoxin is regulated by adenine nucleotides. *Infect. Immun*. **59,** 1552–1557.

Giannella, R.A. (1995). *Escherichia coli* heat-stable enterotoxins, guanylins, and their receptors: what are they and what do they do? *J. Lab. Clin. Med*. **125,** 173–181.

Giannella, R.A., Luttrell, M. and Thompson, M. (1983). Binding of *Escherichia coli* heat-stable enterotoxin to receptors on rat intestinal cells. *Am. J. Physiol*. **245,** G492–498.

Goldstein, J.L., Sahi, J., Bhuva, M., Layden, T.J. and Rao, M.C. (1994). *Escherichia coli* heat-stable enterotoxin-mediated colonic $Cl^-$ secretion is absent in cystic fibrosis. *Gastroenterology* **107,** 950–956.

Greenberg, R.N., Guerrant, R.L., Chang, B., Robertson, D.C. and Murad, F. (1982). Inhibition of *Escherichia coli* heat-stable enterotoxin effects on intestinal guanylate cyclase and fluid secretion by quinacrine. *Biochem. Pharmacol*. **31,** 2005–2009.

Greenberg, R.N., Hill, M., Crytzer, J., Krause, W.J., Eber, S.L., Hamra, F.K. and Forte, L.R. (1997). Comparison of effects of uroguanylin, guanylin, and *Escherichia coli* heat-stable enterotoxin STa in mouse intestine and kidney: evidence that uroguanylin is an intestinal natriuretic hormone. *J. Investig. Med*. **45,** 276–282.

Guarino, A., Capano, G., Malamisura, B., Alessio, M., Guandalini, S. and Rubino, A. (1987a). Production of *Escherichia coli* STa-like heat-stable enterotoxin by *Citrobacter freundii* isolated from humans. *J. Clin. Microbiol*. **25,** 110–114.

Guarino, A., Cohen, M., Thompson, M., Dharmsathaphorn, K. and Giannella, R. (1987b). T84 cell receptor binding and guanyl cyclase activation by *Escherichia coli* heat-stable toxin. *Am. J. Physiol*. **253,** G775–780.

Guarino, A., Cohen, M.B. and Giannella, R.A. (1987c). Small and large intestinal guanylate cyclase activity in children: effect of age and stimulation by *Escherichia coli* heat-stable enterotoxin. *Pediatr. Res*. **21,** 551–555.

Guarino, A., Giannella, R. and Thompson, M.R. (1989a). *Citrobacter freundii* produces an 18-amino-acid heat-stable enterotoxin identical to the 18-amino-acid *Escherichia coli* heat-stable enterotoxin (ST Ia). *Infect. Immun*. **57,** 649–652.

Guarino, A., Guandalini, S., Alessio, M., Gentile, F., Tarallo, L., Capano, G., Migliavacca, M. and Rubino, A. (1989b). Characteristics and mechanism of action of a heat-stable enterotoxin produced by *Klebsiella pneumoniae* from infants with secretory diarrhea. *Pediatr. Res*. **25,** 514–518.

Gyles, C.L. (1994). Escherichia coli enterotoxins. In: Escherichia coli *in Domestic Animals and Human.*, ed. C.L. Gyles, Wallingford, U.K.: CABI International, 337–364.

Gyles, C.L., Palchaudhuri, S. and Maas, W.K. (1977). Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. *Science* **198,** 198–199.

Hakki, S., Robertson, D.C. and Waldman, S.A. (1993). A 56 kDa binding protein for *Escherichia coli* heat-stable enterotoxin isolated from the cytoskeleton of rat intestinal membrane does not possess guanylate cyclase activity. *Biochim. Biophys. Acta* **1152,** 1–8.

Hamra, F.K., Forte, L.R., Eber, S.L., Pidhorodeckyj, N.V., Krause, W.J., Freeman, R.H., Chin, D.T., Tompkins, J.A., Fok, K.F., Smith, C.E. and *et al*. (1993). Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proc. Natl. Acad. Sci. USA* **90,** 10464–10468.

Harnett, N.M. and Gyles, C.L. (1985). Enterotoxin plasmids in bovine and porcine enterotoxigenic *Escherichia coli* of O groups 9, 20, 64, and 101. *Can. J. Comp. Med*. **49,** 79–87.

Henderson, I.R., Hicks, S., Navarro-Garcia, F., Elias, W.P., Philips, A.D. and Nataro, J.P. (1999). Involvement of the enteroaggregative *Escherichia coli* plasmid-encoded toxin in causing human intestinal damage. *Infect. Immun*. **67,** 5338–5344.

Hidaka, Y., Kubota, H., Yoshimura, S., Ito, H., Takeda, Y. and Shimonishi, Y. (1988). Disulphide linkages in a heat-stable enterotoxin (STp) produced by a porcine strain of enterotoxigenic *Escherichia coli*. *Bull. Chem. Soc. Jpn* **61,** 1265–1271.

Hidaka, Y., Ohmori, K., Wada, A., Ozaki, H., Ito, H., Hirayama, T., Takeda, Y. and Shimonishi, Y. (1991). Synthesis and biological properties of carba-analogs of heat-stable enterotoxin (ST) produced by enterotoxigenic *Escherichia coli*. *Biochem. Biophys. Res. Commun* **176,** 958–965.

Hirayama, T., Wada, A., Iwata, N., Takasaki, S., Shimonishi, Y. and Takeda, Y. (1992). Glycoprotein receptors for a heat-stable enterotoxin (STh) produced by enterotoxigenic *Escherichia coli*. *Infect. Immun*. **60,** 4213–4220.

Hitotsubashi, S., Akagi, M., Saitou, A., Yamanaka, H., Fujii, Y. and Okamoto, K. (1992a). Action of *Escherichia coli* heat-stable enterotoxin II on isolated sections of mouse ileum. *FEMS Microbiol. Lett*. **69,** 249–252.

Hitotsubashi, S., Fujii, Y., Yamanaka, H. and Okamoto, K. (1992b). Some properties of purified *Escherichia coli* heat-stable enterotoxin II. *Infect. Immun*. **60,** 4468–4474.

Huang, X., Yoshino, K., Nakao, H. and Takeda, T. (1997). Nucleotide sequence of a gene encoding the novel *Yersinia enterocolitica* heat-stable enterotoxin that includes a pro-region-like sequence in its mature toxin molecule. *Microb. Pathog*. **22,** 89–97.

Hughes, J.M., Murad, F., Chang, B. and Guerrant, R.L. (1978). Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. *Nature* **271,** 755–756.

Hughes, M., Crane, M.R., Thomas, B.R., Robertson, D., Gazzano, D., O'Hanley, P. and Waldman, S.A. (1992). Affinity purification of functional receptors for *Escherichia coli* heat-stable enterotoxin from rat intestine. *Biochemistry* **31,** 12–26.

Hugues, M., Crane, M., Hakki, S., O'Hanley, P. and Waldman, S.A. (1991). Identification and characterization of a new family of high-affinity receptors for *Escherichia coli* heat-stable enterotoxin in rat intestinal membranes. *Biochemistry* **30,** 10738–10745.

Huott, P.A., Liu, W., McRoberts, J.A., Giannella, R.A. and Dharmsathaphorn, K. (1988). Mechanism of action of *Escherichia coli* heat stable enterotoxin in a human colonic cell line. *J. Clin. Invest*. **82,** 514–523.

Ibrahim, A., Liesack, W. and Stackebrandt, E. (1992). Differentiation between pathogenic and non-pathogenic *Yersinia enterocolitica* strains by colony hybridization with a PCR-mediated digoxigenin-dUTP-labeled probe. *Mol. Cell. Probes* **6,** 163–171.

Ikemura, H., Yoshimura, S., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T. and Miwatani, T. (1984). Synthesis of heat-stable enterotoxin (STh) produced by a human strain SK-1 of enterotoxigenic *Escherichia coli. Bull. Chem. Soc. Jpn* **57,** 2543–2549.

Inoue, R., Okamoto, K., Moriyama, T., Takahashi, T., Shimuzu, K. and Miyama, A. (1983). Synthesis of *Yersinia enterolitica* ST on cyclic guanosine 3′, 5′-monophosphate levels in mouse intestines and cultured cells. *Microbiol. Immunol*. **27,** 159–166.

Itoh, Y., Nagano, I., Kunishima, M. and Ezaki, T. (1997). Laboratory investigation of enteroaggregative *Escherichia coli* O untypeable:H10 associated with a massive outbreak of gastrointestinal illness. *J. Clin. Microbiol*. **35,** 2546–2550.

Ivens, K., Gazzano, H., O'Hanley, P. and Waldman, S.A. (1990). Heterogeneity of intestinal receptors for *Escherichia coli* heat-stable enterotoxin. *Infect. Immun*. **58,** 1817–1820.

Katwa, L.C., Parker, C.D., Dybing, J.K. and White, A.A. (1992). Nucleotide regulation of heat-stable enterotoxin receptor binding and of guanylate cyclase activation. *Biochem. J*. **283,** 727–735.

Kawano, K., Yamada, T., Yagi, T. and Ito, K. (1998). Detection of enteroaggregative *Escherichia coli* sporadic diarrhea patients. *Kansenshogaku Zasshi* **72,** 1275–1282.

Kita, T., Smith, C.E., Fok, K.F., Duffin, K.L., Moore, W.M., Karabatsos, P.J., Kachur, J.F., Hamra, F.K., Pidhorodeckyj, N.V., Forte, L.R. and *et al*. (1994). Characterization of human uroguanylin: a member of the guanylin peptide family. *Am. J. Physiol*. **266,** F342–348.

Knoop, F.C., Owens, M., Marcus, J.N. and Murphy, B. (1991). Elevation of calcium in rat enterocytes by *Escherichia coli* heat-stable (STa) enterotoxin. *Curr. Microbiol*. **23,** 291–296.

Krause, W.J., Cullingford, G.L., Freeman, R.H., Eber, S.L., Richardson, K.C., Fok, K.F., Currie, M.G. and Forte, L.R. (1994). Distribution of heat-stable enterotoxin/guanylin receptors in the intestinal tract of man and other mammals. *J. Anat*. **184,** 407–417.

Kuhn, M., Adermann, K., Jahne, J., Forssmann, W.G. and Rechkemmer, G. (1994). Segmental differences in the effects of guanylin and *Escherichia coli* heat-stable enterotoxin on $Cl^-$ secretion in human gut. *J. Physiol*. **479,** 433–440.

Kuhn, M., Raida, M., Adermann, K., Schulz-Knappe, P., Gerzer, R., Heim, J.M. and Forssmann, W.G. (1993). The circulating bioactive form of human guanylin is a high molecular weight peptide (10.3 kDa). *FEBS Lett*. **318,** 205–209.

Lai, X.H., Wang, S.Y. and Uhlin, B.E. (1999). Expression of cytotoxicity by potential pathogens in the standard *Escherichia coli* collection of reference (ECOR) strains. *Microbiology* **145,** 3295–3303.

Law, D. and Chart, H. (1998). Enteroaggregative *Escherichia coli. J. Appl. Microbiol*. **84,** 685–697.

Levine, M.M., Prado, V., Robins-Browne, R., Lior, H., Kaper, J.B., Moseley, S.L., Gicquelais, K., Nataro, J.P., Vial, P. and Tall, B. (1988). Use of DNA probes and HEp-2 cell adherence assay to detect diarrheagenic *Escherichia coli. J. Infect. Dis*. **158,** 224–228.

Lin, M., Nairn, A.C. and Guggino, S.E. (1992). cGMP-dependent protein kinase regulation of a chloride channel in T84 cells. *Am. J. Physiol*. **262,** C1304–1312.

Lundgren, O. (2004). Interface between the intestinal environment and the nervous system. *Gut* **53** *Suppl 2,* ii16–18.

Madsen, G.L. and Knoop, F.C. (1978). Inhibition of the secretory activity of *Escherichia coli* heat-stable enterotoxin by indomethacin. *Infect. Immun*. **22,** 143–147.

Mallard, K.E. and Desmarchelier, P.M. (1995). Detection of heat-stable enterotoxin genes among Australian *Vibrio cholerae* O1 strains. *FEMS Microbiol. Lett*. **127,** 111–115.

Mann, E.A., Cohen, M.B. and Giannella, R.A. (1993). Comparison of receptors for *Escherichia coli* heat-stable enterotoxin: novel receptor present in IEC-6 cells. *Am. J. Physiol*. **264,** G172–178.

Mann, E.A., Jump, M.L., Wu, J., Yee, E. and Giannella, R.A. (1997). Mice lacking the guanylyl cyclase C receptor are resistant to STa-induced intestinal secretion. *Biochem. Biophys. Res. Commun*. **239,** 463–466.

Markert, T., Vaandrager, A.B., Gambaryan, S., Pohler, D., Hausler, C., Walter, U., De Jonge, H.R., Jarchau, T. and Lohmann, S.M. (1995). Endogenous expression of type II cGMP-dependent protein kinase mRNA and protein in rat intestine. Implications for cystic fibrosis transmembrane conductance regulator. *J. Clin. Invest*. **96,** 822–830.

Mathias, J.R., Nogueira, J., Martin, J.L., Carlson, G.M. and Giannella, R.A. (1982). *Escherichia coli* heat-stable toxin: its effect on motility of the small intestine. *Am. J. Physiol*. **242,** G360–363.

Matthews, J.B., Awtrey, C.S., Thompson, R., Hung, T., Tally, K.J. and Madara, J.L. (1993). Na(+)-K(+)-2$Cl^-$ cotransport and $Cl^-$ secretion evoked by heat-stable enterotoxin is microfilament dependent in T84 cells. *Am. J. Physiol*. **265,** G370–378.

McVeigh, A., Fasano, A., Scott, D.A., Jelacic, S., Moseley, S.L., Robertson, D.C. and Savarino, S.J. (2000). IS1414, an *Escherichia coli* insertion sequence with a heat-stable enterotoxin gene embedded in a transposase-like gene. *Infect. Immun*. **68,** 5710–5715.

Ménard, L.P. and Dubreuil, J.D. (2002). Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 (EAST1): a new toxin with an old twist. *Crit. Rev. Microbiol*. **28,** 43–60.

Ménard, L.P., Lussier, J.G., Lépine, F., Paiva de Sousa, C. and Dubreuil, J.D. (2004). Expression, purification, and biochemical characterization of enteroaggregative *Escherichia coli* heat-stable enterotoxin 1. *Protein Expr. Purif*. **33,** 223–231.

Mikulskis, A.V., Delor, I., Thi, V.H. and Cornelis, G.R. (1994). Regulation of the *Yersinia enterocolitica* enterotoxin *Yst* gene. Influence of growth phase, temperature, osmolarity, pH, and bacterial host factors. *Mol. Microbiol*. **14,** 905–915.

Monteiro-Neto, V., Campos, L.C., Ferreira, A.J., Gomes, T.A. and Trabulsi, L.R. (1997). Virulence properties of *Escherichia coli* O111:H12 strains. *FEMS Microbiol. Lett*. **146,** 123–128.

Morris, J.G., Jr., Takeda, T., Tall, B.D., Losonsky, G.A., Bhattacharya, S.K., Forrest, B.D., Kay, B.A. and Nishibuchi, M. (1990). Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. *J. Clin. Invest*. **85,** 697–705.

Nair, G.B. and Takeda, Y. (1998). The heat-stable enterotoxins. *Microb. Pathog*. **24,** 123–131.

Nataro, J.P., Deng, Y., Cookson, S., Cravioto, A., Savarino, S.J., Guers, L.D., Levine, M.M. and Tacket, C.O. (1995). Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. *J. Infect. Dis*. **171,** 465–468.

Nataro, J.P., Hicks, S., Phillips, A.D., Vial, P.A. and Sears, C.L. (1996). T84 cells in culture as a model for enteroaggregative *Escherichia coli* pathogenesis. *Infect. Immun*. **64,** 4761–4768.

Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli. Clin. Microbiol. Rev*. **11,** 142–201.

Nataro, J.P., Steiner, T. and Guerrant, R.L. (1998). Enteroaggregative *Escherichia coli. Emerg. Infect. Dis*. **4,** 251–261.

Nataro, J.P., Yikang, D., Giron, J.A., Savarino, S.J., Kothary, M.H. and Hall, R. (1993). Aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli* requires two unlinked plasmid regions. *Infect. Immun*. **61,** 1126–1131.

Nishikawa, Y., Ogasawara, J., Helander, A. and Haruki, K. (1999). An outbreak of gastroenteritis in Japan due to *Escherichia coli* O166. *Emerg. Infect. Dis*. **5,** 300.

Nzegwu, H.C. and Levin, R.J. (1996). Luminal capsaicin inhibits fluid secretion induced by enterotoxin *E. coli* STa, but not by carbachol, *in vivo* in rat small and large intestine. *Exp. Physiol*. **81,** 313–315.

Ogawa, A., Kato, J., Watanabe, H., Suzuki, K., Dohi, S. and Takeda, T. (1990). Cloning and nucleotide sequence determination of a heat-stable enterotoxin gene from *Vibrio cholerae* non-O1. *Jpn J. Med. Sci. Biol*. **43,** 255.

Ogawa, A. and Takeda, T. (1993). The gene encoding the heat-stable enterotoxin of *Vibrio cholerae* is flanked by 123-base pair direct repeats. *Microbiol. Immunol*. **37,** 607–616.

Okamoto, K., Baba, T., Yamanaka, H., Akashi, N. and Fujii, Y. (1995). Disulfide bond formation and secretion of *Escherichia coli* heat-stable enterotoxin II. *J. Bacteriol*. **177,** 4579–4586.

Okamoto, K. and Takahara, M. (1990). Synthesis of *Escherichia coli* heat-stable enterotoxin STp as a pre-pro form and role of the pro sequence in secretion. *J. Bacteriol*. **172,** 5260–5265.

Okamoto, K., Yukitake, J., Kawamoto, Y. and Miyama, A. (1987). Substitutions of cysteine residues of *Escherichia coli* heat-stable enterotoxin by oligonucleotide-directed mutagenesis. *Infect. Immun*. **55,** 2121–2125.

Ozaki, H., Sato, T., Kubota, H., Hata, Y., Katsube, Y. and Shimonishi, Y. (1991). Molecular structure of the toxin domain of heat-stable enterotoxin produced by a pathogenic strain of *Escherichia coli*. A putative binding site for a binding protein on rat intestinal epithelial cell membranes. *J. Biol. Chem*. **266,** 5934–5941.

Pai, C.H. and Mors, V. (1978). Production of enterotoxin by *Yersinia enterolitica* gastroenteritis. *Infect. Immun*. **22,** 334–338.

Paiva de Sousa, C. and Dubreuil, J.D. (2001). Distribution and expression of the *astA* gene (EAST1 toxin) in *Escherichia coli* and *Salmonella. Int. J. Med. Microbiol*. **291,** 15–20.

Peruski, J., L.F. and Peruski, A.H. (1997). EAST1 of *E. coli* is a heat-stable enterotoxin that is evolutionarily distinct from the enterotoxin family. *97th General Meeting of the American Society for Microbiology*, **319**.

Pfeifer, A., Aszodi, A., Seidler, U., Ruth, P., Hofmann, F. and Fassler, R. (1996). Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II. *Science* **274,** 2082–2086.

Ramamurthy, T., Albert, M.J., Huq, A., Colwell, R.R., Takeda, Y., Takeda, T., Shimada, T., Mandal, B.K. and Nair, G.B. (1994). *Vibrio mimicus* with multiple toxin types isolated from human and environmental sources. *J. Med. Microbiol*. **40,** 194–196.

Ramamurthy, T., Yoshino, K., Huang, X., Balakrish Nair, G., Carniel, E., Maruyama, T., Fukushima, H. and Takeda, T. (1997). The novel heat-stable enterotoxin subtype gene (*ystB*) of *Yersinia enterocolitica*: nucleotide sequence and distribution of the *yst* genes. *Microb. Pathog*. **23,** 189–200.

Rasheed, J.K., Guzman-Verduzco, L.M. and Kupersztoch, Y.M. (1990). Two precursors of the heat-stable enterotoxin of *Escherichia coli*: evidence of extracellular processing. *Mol. Microbiol*. **4,** 265–273.

Rich, C., Favre-Bonte, S., Sapena, F., Joly, B. and Forestier, C. (1999). Characterization of enteroaggregative *Escherichia coli* isolates. *FEMS Microbiol. Lett*. **173,** 55–61.

Rolfe, V. and Levin, R.J. (1994). Enterotoxin *Escherichia coli* STa activates a nitric oxide-dependent myenteric plexus secretory reflex in the rat ileum. *J. Physiol*. **475,** 531–537.

Roussel, A.J., Woode, G.N., Waldron, R.C., Sriranganathan, N. and Jones, M.K. (1992). Myoelectric activity of the small intestine in enterotoxin-induced diarrhea of calves. *Am. J. Vet. Res*. **53,** 1145–1148.

Rudner, X.L., Mandal, K.K., de Sauvage, F.J., Kindman, L.A. and Almenoff, J.S. (1995). Regulation of cell signaling by the cytoplasmic domains of the heat-stable enterotoxin receptor: identification of autoinhibitory and activating motifs. *Proc. Natl. Acad. Sci. USA* **92,** 5169–5173.

Sanchez, J., Solorzano, R.M. and Holmgren, J. (1993). Extracellular secretion of STa heat-stable enterotoxin by *Escherichia coli* after fusion to a heterologous leader peptide. *FEBS Lett*. **330,** 265–269.

Sato, T., Ozaki, H., Hata, Y., Kitagawa, Y., Katsube, Y. and Shimonishi, Y. (1994). Structural characteristics for biological activity of heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli*: x-ray crystallography of weakly toxic and non-toxic analogs. *Biochemistry* **33,** 8641–8650.

Savarino, S.J., Fasano, A., Robertson, D.C. and Levine, M.M. (1991). Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an *in vitro* rabbit intestinal model. *J. Clin. Invest*. **87,** 1450–1455.

Savarino, S.J., Fasano, A., Watson, J., Martin, B.M., Levine, M.M., Guandalini, S. and Guerry, P. (1993). Enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 represents another subfamily of *E. coli* heat-stable toxin. *Proc. Natl. Acad. Sci. USA* **90,** 3093–3097.

Savarino, S.J., Fox, P., Deng, Y. and Nataro, J.P. (1994). Identification and characterization of a gene cluster mediating enteroaggregative *Escherichia coli* aggregative adherence fimbria I biogenesis. *J. Bacteriol*. **176,** 4949–4957.

Savarino, S.J., McVeigh, A., Watson, J., Cravioto, A., Molina, J., Echeverria, P., Bhan, M.K., Levine, M.M. and Fasano, A. (1996). Enteroaggregative *Escherichia coli* heat-stable enterotoxin is not restricted to enteroaggregative *E. coli*. *J. Infect. Dis*. **173,** 1019–1022.

Scheving, L.A. and Chong, K.M. (1997). Differential processing of guanylyl cyclase C along villus-crypt axis of rat small intestine. *Am. J. Physiol*. **272,** C1995–2004.

Schulz, S., Green, C.K., Yuen, P.S. and Garbers, D.L. (1990). Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* **63,** 941–948.

Schulz, S., Yuen, P.S. and Garbers, D.L. (1991). The expanding family of guanylyl cyclases. *Trends Pharmacol. Sci*. **12,** 116–120.

Sears, C.L. and Kaper, J.B. (1996). Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol. Rev*. **60,** 167–215.

Sekizaki, T., Akashi, H. and Terakado, N. (1985). Nucleotide sequences of the genes for *Escherichia coli* heat-stable enterotoxin I of bovine, avian, and porcine origins. *Am. J. Vet. Res*. **46,** 909–912.

Shimonishi, Y., Hidaka, Y., Koizumi, M., Hane, M., Aimoto, S., Takeda, T., Miwatani, T. and Takeda, Y. (1987). Mode of disulfide bond formation of a heat-stable enterotoxin (STh) produced by a human strain of enterotoxigenic *Escherichia coli*. *FEBS Lett*. **215,** 165–170.

Sjostrom, M., Wold, S., Wieslander, A. and Rilfors, L. (1987). Signal peptide amino acid sequences in *Escherichia coli* contain information related to final protein localization. A multivariate data analysis. *EMBO J*. **6,** 823–831.

So, M. and McCarthy, B.J. (1980). Nucleotide sequence of the bacterial transposon Tn1681 encoding a heat-stable (ST) toxin and its identification in enterotoxigenic *Escherichia coli* strains. *Proc. Natl. Acad. Sci. USA* **77,** 4011–4015.

Sommerfelt, H., Haukanes, B.I., Kalland, K.H., Svennerholm, A.M., Sanchez, J. and Bjorvatn, B. (1989). Mechanism of spontaneous loss of heat-stable toxin (STa) production in enterotoxigenic *Escherichia coli*. *Apmis* **97,** 436–440.

Sprangler, B.D. (1992). Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev*. **56,** 622–647.

Staples, S.J., Asher, S.E. and Giannella, R.A. (1980). Purification and characterization of heat-stable enterotoxin produced by a strain of *E. coli* pathogenic for man. *J. Biol. Chem*. **255,** 4716–4721.

Stephan, R. and Untermann, F. (1999). Virulence factors and phenotypical traits of verotoxin-producing *Escherichia coli* strains isolated from asymptomatic human carriers. *J. Clin. Microbiol*. **37,** 1570–1572.

Stieglitz, H., Cervantes, L., Robledo, R., Fonseca, R., Covarrubias, L., Bolivar, F. and Kupersztoch, Y.M. (1988). Cloning, sequencing,

and expression in Ficoll-generated minicells of an *Escherichia coli* heat-stable enterotoxin gene. *Plasmid* **20,** 42–53.

Swenson, E.S., Mann, E.A., Jump, M.L., Witte, D.P. and Giannella, R.A. (1996). The guanylin/STa receptor is expressed in crypts and apical epithelium throughout the mouse intestine. *Biochem. Biophys. Res. Commun*. **225,** 1009–1014.

Takao, T., Hitouji, T., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Takeda, Y. and Miwatani, T. (1983). Amino acid sequence of a heat-stable enterotoxin isolated from enterotoxigenic *Escherichia coli* strain 18D. *FEBS Lett*. **152,** 1–5.

Takao, T., Shimonishi, Y., Kobayashi, M., Nishimura, O., Arita, M., Takeda, T., Honda, T. and Miwatani, T. (1985a). Amino acid sequence of heat-stable enterotoxin produced by *Vibrio cholerae* non-01. *FEBS Lett*. **193,** 250–254.

Takao, T., Tominaga, N., Shimonishi, Y., Hara, S., Inoue, T. and Miyama, A. (1984). Primary structure of heat-stable enterotoxin produced by *Yersinia enterocolitica. Biochem. Biophys. Res. Commun*. **125,** 845–851.

Takao, T., Tominaga, N., Yoshimura, S., Shimonishi, Y., Hara, S., Inoue, T. and Miyama, A. (1985b). Isolation, primary structure, and synthesis of heat-stable enterotoxin produced by *Yersinia enterocolitica. Eur. J. Biochem*. **152,** 199–206.

Takeda, T., Takeda, Y., Aimoto, S., Takao, T., Ikemura, H., Shimonishi, Y. and Miwatani, T. (1983). Neutralization of activity of two different heat-stable enterotoxins (STh and STp) of enterotoxigenic *Escherichia coli* by homologous and heterologous antisera. *FEMS Microbiol. Lett*. **20,** 357–359.

Takeda, T., Nair, G.B., Suzuki, K. and Shimonishi, Y. (1990). Production of a monoclonal antibody to *Vibrio cholerae* non-O1 heat-stable enterotoxin (ST), which is cross-reactive with *Yersinia enterocolitica* ST. *Infect. Immun*. **58,** 2755–2759.

Takeda, T., Peina, Y., Ogawa, A., Dohi, S., Abe, H., Nair, G.B. and Pal, S.C. (1991a). Detection of heat-stable enterotoxin in a cholera toxin, gene-positive strain of *Vibrio cholerae* O1. *FEMS Microbiol. Lett*. **64,** 23–27.

Takeda, Y., Yamasaki, S., Hirayama, T. and Shimonishi, Y. (1991b). Heat-stable enterotoxins produced by enteric bacteria. In: *Molecular Pathogenesis of Gastrointestinal Infections*., vol. FEMS Symposium 58, ed. T. Wadstrom, Makela, P.H., Svennerholm, A.M. and Wolf-Watz, H., New-York: Plenum Press, 125–128.

Thomas, D.D. and Knoop, F.C. (1982). The effect of calcium and prostaglandin inhibitors on the intestinal fluid response to heat-stable enterotoxin of *Escherichia coli. J. Infect. Dis*. **145,** 141–147.

Thompson, M.R. and Giannella, R.A. (1985). Revised amino acid sequence for a heat-stable enterotoxin produced by an *Escherichia coli* strain (18D) that is pathogenic for humans. *Infect. Immun*. **47,** 834–836.

Thompson, M.R. and Giannella, R.A. (1990). Different cross-linking agents identify distinctly different putative *Escherichia coli* heat-stable enterotoxin rat intestinal cell receptor proteins. *J. Recept. Res*. **10,** 97–117.

Tien, X.Y., Brasitus, T.A., Kaetzel, M.A., Dedman, J.R. and Nelson, D.J. (1994). Activation of the cystic fibrosis transmembrane conductance regulator by cGMP in the human colonic cancer cell line, Caco-2. *J. Biol. Chem*. **269,** 51–54.

Tzipori, S., Montanaro, J., Robins-Browne, R.M., Vial, P., Gibson, R. and Levine, M.M. (1992). Studies with enteroaggregative *Escherichia coli* in the gnotobiotic piglet gastroenteritis model. *Infect. Immun*. **60,** 5302–5306.

Uzzau, S. and Fasano, A. (2000). Cross-talk between enteric pathogens and the intestine. *Cell. Microbiol*. **2,** 83–89.

Vaandrager, A.B., Bot, A.G. and De Jonge, H.R. (1997a). Guanosine 3′,5′-cyclic monophosphate-dependent protein kinase II mediates heat-stable, enterotoxin-provoked chloride secretion in rat intestine. *Gastroenterology* **112,** 437–443.

Vaandrager, A.B., Edixhoven, M., Bot, A.G., Kroos, M.A., Jarchau, T., Lohmann, S., Genieser, H.G. and de Jonge, H.R. (1997b). Endogenous type II cGMP-dependent protein kinase exists as a dimer in membranes and can be functionally distinguished from the type I isoforms. *J. Biol. Chem*. **272,** 11816–11823.

Vaandrager, A.B., Ehlert, E.M., Jarchau, T., Lohmann, S.M. and de Jonge, H.R. (1996). N-terminal myristoylation is required for membrane localization of cGMP-dependent protein kinase type II. *J. Biol. Chem*. **271,** 7025–7029.

Vaandrager, A.B., Schulz, S., De Jonge, H.R. and Garbers, D.L. (1993a). Guanylyl cyclase C is an N-linked glycoprotein receptor that accounts for multiple heat-stable enterotoxin-binding proteins in the intestine. *J. Biol. Chem*. **268,** 2174–2179.

Vaandrager, A.B., van der Wiel, E. and de Jonge, H.R. (1993b). Heat-stable enterotoxin activation of immunopurified guanylyl cyclase C. Modulation by adenine nucleotides. *J. Biol. Chem*. **268,** 19598–19603.

Vaandrager, A.B., van der Wiel, E., Hom, M.L., Luthjens, L.H. and de Jonge, H.R. (1994). Heat-stable enterotoxin receptor/guanylyl cyclase C is an oligomer consisting of functionally distinct subunits, which are non-covalently linked in the intestine. *J. Biol. Chem*. **269,** 16409–16415.

Vajanaphanich, M., Kachintorn, U., Barrett, K.E., Cohn, J.A., Dharmsathaphorn, K. and Traynor-Kaplan, A. (1993). Phosphatidic acid modulates $Cl^-$ secretion in T84 cells: varying effects depending on mode of stimulation. *Am. J. Physiol*. **264,** C1210–1218.

Vila, J., Gene, A., Vargas, M., Gascon, J., Latorre, C. and Jimenez de Anta, M.T. (1998). A case-control study of diarrhea in children caused by *Escherichia coli* producing heat-stable enterotoxin (EAST-1). *J. Med. Microbiol*. **47,** 889–891.

Vila, J., Vargas, M., Henderson, I.R., Gascon, J. and Nataro, J.P. (2000). Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. *J. Infect. Dis*. **182,** 1780–1783.

Wada, A., Hasegawa, M., Matsumoto, K., Niidome, T., Kawano, Y., Hidaka, Y., Padilla, P.I., Kurazono, H., Shimonishi, Y. and Hirayama, T. (1996). The significance of Ser1029 of the heat-stable enterotoxin receptor (STaR): relation of STa-mediated guanylyl cyclase activation and signaling by phorbol myristate acetate. *FEBS Lett*. **384,** 75–77.

Wada, A., Hirayama, T., Kitao, S., Fujisawa, J., Hidaka, Y. and Shimonishi, Y. (1994). Pig intestinal membrane-bound receptor (guanylyl cyclase) for heat-stable enterotoxin: cDNA cloning, functional expression, and characterization. *Microbiol. Immunol*. **38,** 535–541.

Waldman, S.A., Kuno, T., Kamisaki, Y., Chang, L.Y., Gariepy, J., O'Hanley, P., Schoolnik, G. and Murad, F. (1986). Intestinal receptor for heat-stable enterotoxin of *Escherichia coli* is tightly coupled to a novel form of particulate guanylate cyclase. *Infect. Immun*. **51,** 320–326.

Waldman, S.A. and O'Hanley, P. (1989). Influence of a glycine or proline substitution on the functional properties of a 14-amino-acid analog of *Escherichia coli* heat-stable enterotoxin. *Infect. Immun*. **57,** 2420–2424.

Wanke, C.A., Mayer, H., Weber, R., Zbinden, R., Watson, D.A. and Acheson, D. (1998). Enteroaggregative *Escherichia coli* as a potential cause of diarrheal disease in adults infected with human immunodeficiency virus. *J. Infect. Dis*. **178,** 185–190.

Weikel, C.S., Spann, C.L., Chambers, C.P., Crane, J.K., Linden, J. and Hewlett, E.L. (1990). Phorbol esters enhance the cyclic GMP response of T84 cells to the heat-stable enterotoxin of *Escherichia coli* (STa). *Infect. Immun*. **58,** 1402–1407.

Weikel, C.S. and Guerrant, R.L. (1985). STb enterotoxin of *Escherichia coli*: cyclic nucleotide-independent secretion. In: *Microbial Toxins and Diarrhoeal Disease*, London: Pitman, 94–115.

Whipp, S.C. (1990). Assay for enterotoxigenic *Escherichia coli* heat-stable toxin B in rats and mice. *Infect. Immun*. **58,** 930–934.

Whipp, S.C. (1991). Intestinal responses to enterotoxigenic *Escherichia coli* heat-stable toxin B in non-porcine species. *Am. J. Vet. Res*. **52,** 734–737.

Wiegand, R.C., Kato, J., Huang, M.D., Fok, K.F., Kachur, J.F. and Currie, M.G. (1992). Human guanylin: cDNA isolation, structure, and activity. *FEBS Lett*. **311,** 150–154.

Wolf, M.K. (1997). Occurrence, distribution, and associations of O and H serogroups, colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli. Clin. Microbiol. Rev*. **10,** 569–584.

Yamamoto, T. and Echeverria, P. (1996). Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E. coli* strains pathogenic for humans. *Infect. Immun*. **64,** 1441–1445.

Yamamoto, T. and Nakazawa, M. (1997). Detection and sequences of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene in enterotoxigenic *E. coli* strains isolated from piglets and calves with diarrhea. *J. Clin. Microbiol*. **35,** 223–227.

Yamamoto, T. and Taneike, I. (2000). The sequences of enterohemorrhagic *Escherichia coli* and *Yersinia pestis* that are homologous to the enteroaggregative *E. coli* heat-stable enterotoxin gene: cross-species transfer in evolution. *FEBS Lett*. **472,** 22–26.

Yamamoto, T., Wakisaka, N. and Nakae, T. (1997a). A novel cryohemagglutinin associated with adherence of enteroaggregative *Escherichia coli. Infect. Immun*. **65,** 3478–3484.

Yamamoto, T., Wakisaka, N., Sato, F. and Kato, A. (1997b). Comparison of the nucleotide sequence of enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 genes among diarrhea-associated *Escherichia coli. FEMS Microbiol. Lett*. **147,** 89–95.

Yamanaka, H., Fuke, Y., Hitotsubashi, S., Fujii, Y. and Okamoto, K. (1993). Functional properties of pro region of *Escherichia coli* heat-stable enterotoxin. *Microbiol. Immunol*. **37,** 195–205.

Yamanaka, H., Kameyama, M., Baba, T., Fujii, Y. and Okamoto, K. (1994). Maturation pathway of *Escherichia coli* heat-stable enterotoxin I: requirement of DsbA for disulfide bond formation. *J. Bacteriol*. **176,** 2906–2913.

Yamanaka, H., Nomura, T., Fujii, Y. and Okamoto, K. (1997). Extracellular secretion of *Escherichia coli* heat-stable enterotoxin I across the outer membrane. *J. Bacteriol*. **179,** 3383–3390.

Yamanaka, H., Nomura, T., Fujii, Y. and Okamoto, K. (1998a). Need for TolC, an *Escherichia coli* outer membrane protein, in the secretion of heat-stable enterotoxin I across the outer membrane. *Microb. Pathog*. **25,** 111–120.

Yamanaka, H., Nomura, T. and Okamoto, K. (1998b). Involvement of glutamic acid residue at position 7 in the formation of the intramolecular disulfide bond of *Escherichia coli* heat-stable enterotoxin Ip *in vivo. Microb. Pathog*. **24,** 145–154.

Yamanaka, H. and Okamoto, K. (1996). Amino acid residues in the pro region of *Escherichia coli* heat-stable enterotoxin I that affect efficiency of translocation across the inner membrane. *Infect. Immun*. **64,** 2700–2708.

Yamasaki, S., Hidaka, Y., Ito, H., Takeda, Y. and Shimonishi, Y. (1988). Structure requirements for the spatial structure and toxicity of heat-stable enterotoxin (STh) of enterotoxigenic *Escherichia coli. Bull. Chem. Soc. Jpn* **61,** 1701–1706.

Yang, Y., Gao, Z., Guzman-Verduzco, L.M., Tachias, K. and Kupersztoch, Y.M. (1992). Secretion of the STA3 heat-stable enterotoxin of *Escherichia coli*: extracellular delivery of Pro-STA is accomplished by either Pro or STA. *Mol. Microbiol*. **6,** 3521–3529.

Yoshimura, S., Ikemura, H., Watanabe, H., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., Miwatani, T. and Takeda, Y. (1985). Essential structure for full enterotoxigenic activity of heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli. FEBS Lett*. **181,** 138–142.

Yoshino, K., Huang, X., MIyachi, M., Hong, Y.-M., Tako, T., Nakao, H., Takeda, T. and Simonishi, Y. (1994). Amino acid sequence of a novel heat-stable enterotoxin produced by a *yst* gene-negative strain of *Yersinia enterolitica. Lett. Peptide Sci*. **1,** 95–105.

Yoshino, K., Miyachi, M., Takao, T., Bag, P.K., Huang, X., Nair, G.B., Takeda, T. and Shimonishi, Y. (1993). Purification and sequence determination of heat-stable enterotoxin elaborated by a cholera toxin-producing strain of *Vibrio cholerae* O1. *FEBS Lett*. **326,** 83–86.

Yoshino, K., Takao, T., Huang, X., Murata, H., Nakao, H., Takeda, T. and Shimonishi, Y. (1995). Characterization of a highly toxic, large molecular size heat-stable enterotoxin produced by a clinical isolate of *Yersinia enterocolitica. FEBS Lett*. **362,** 319–322.

Yuan, P., Ogawa, A., Ramamurthy, T., Nair, G.B., Shimada, T., Shinoda, S. and Takeda, T. (1994). *Vibrio mimicus* are the reservoirs of the heat-stable enterotoxin gene (*nag-st*) among species of the genus *Vibrio. World J. Microbiol. Biotechnol*. **10,** 59–63.

Zhou, Z., Ogasawara, J., Nishikawa, Y., Seto, Y., Helander, A., Hase, A., Iritani, N., Nakamura, H., Arikawa, K., Kai, A., Kamata, Y., Hoshi, H. and Haruki, K. (2002). An outbreak of gastroenteritis in Osaka, Japan due to *Escherichia coli* serogroup O166:H15 that had a coding gene for enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1). *Epidemiol. Infect*. **128,** 363–371.

SECTION IV

# SUPERANTIGENIC TOXINS

CHAPTER

# 49

# What are superantigens?

*Joseph E. Alouf and Heide Müller-Alouf*

## INITIAL INVESTIGATIONS

Pioneering investigations undertaken during the latter 1980s discovered unexpected immunological properties attributed to the highly mitogenic staphylococcal enterotoxins (SEs) on T lymphocytes. Such findings subsequently opened new research avenues into basic and clinical microbiology as well as immunology, which particulary delved into the domain of bacterial protein toxins. The triggering of lymphocyte proliferation by SEs requires selective and simultaneous binding of these molecules by both major histocompatibility (MHC) class II molecules at the surface of accessory cells (e.g., peripheral blood mononuclear cells) and T cell receptors (TcR) on target lymphocytes. In this respect, SEs appear as functionally bivalent molecules cross-linking TcR and MHC class II molecules in a unique manner. It was shown that the dependence on class II molecules was not due to an immunological "recognition" of SEs as is commonly known for conventional antigens, since there was no restriction by polymorphic determinants of human MHC class II molecules (HLA) and even xenogenic class II molecules (Fleischer and Schrezenmeier, 1988; Fleischer *et al*., 1989; Carlsson *et al*., 1988; Fraser, 1989; Uchiyama *et al*., 1989a; Mollick *et al*., 1989; Scholl *et al*., 1989). In contrast to classical antigens, the T cell response towards SEs showed extensive heterogeneity regarding T cell clonal activation (Fleischer and Schrezenmeier, 1988; Fleischer *et al*., 1989). The target of SEs on T cells was identified as polypeptide segments of the variable β chain (Vβ) region on the TcR with relatively little or no involvement from other parts of the receptor (White *et al*., 1989; Choi *et al*., 1989; Marrack and Kappler, 1990). Moreover, the binding of SEs to MHC class II molecules required no processing and occurred outside of the conventional epitope binding groove (Dellabona *et al*., 1990). These newly identified properties of SEs (Kappler *et al*., 1989) and also staphylococcal toxic shock syndrome toxin 1 (TSST-1) (Uchiyama *et al*. 1989a) led White *et al*. (1989) to coin the term "superantigen" (SAg) to designate these particular properties of SEs and thereafter that of other microbial proteins sharing the same properties (Marrack and Kappler, 1990).

SAg activation of immune system cells was also shown to trigger elevated secretion levels of cytokines such as interferon γ tumor necrosis factor-α (TN F-α) interleukin-1 β, and interleukin-2 (Cavaillon *et al*., 1982; Parsonnet *et al*., 1985; Parsonnet and Gillis, 1988; Jupin *et al*., 1988; Uchiyama *et al*., 1989b; Fast *et al*., 1989; Fischer *et al*., 1990). As described in another section, the *in vivo* release of these effectors may result in various diseases of a severe, acute, and chronic nature.

## MAJOR FUNCTIONAL FEATURES OF SUPERANTIGENS

There is no doubt that the initial seminal studies that led to the superantigen concept have subsequently spurred a wealth of intense research by many laboratories over the last fourteen years. A combination of experimental approaches that include biochemistry, biophysics, microbiology, molecular biology, genomics, immunology, cell biology, crystallography, animal models, and the clinical sciences has facilitated

the considerable progress made towards understanding these fascinating molecules. The state of the art concerning the multifaceted aspects of SAgs, from basic molecular mechanisms to the patient's bed, is presented in the following four chapters (50 to 54) of this volume. In this chapter, we briefly highlight our current knowledge of bacterial SAgs that particularly involve the mechanisms by which these proteins react with immune system cells and the resulting powerful responses by the host, which can corrupt normal immune responses to infection, favor autoimmune processes, and contribute to the pathogenesis of a number of diseases. Recent reviews may help the reader to globally appreciate the recent achievements linked to these molecules and their study (Leung *et al*., 1997; Kotb, 1997; Florkin and Aaldering, 1997; Krakauer, 1999; Alouf *et al*., 1999; Li *et al*., 1998; Fraser *et al*., 2000; McCormick *et al*., 2001b; Müller-Alouf *et al*., 2001a; Llewelyn and Cohen, 2002; Alouf and Müller-Alouf, 2003; Krakauer and Stiles, 2003; Baker and Acharya, 2004; Baker *et al*., 2004; Petersson *et al*., 2004).

The SAgs of viral origin are beyond the scope of this review. Therefore, we refer the reader to articles by Marrack and Kappler, 1990; Huber *et al*. 1996; Conrad *et al*., 1997; Stauffer *et al*., 2001; Llewlyn and Cohen, 2002; Wirth *et al*., 2000; Sutkowski *et al*., 2004.

## INTERACTION OF SAGS WITH IMMUNE SYSTEM CELLS

This interaction leads to the formation of trimolecular complexes via simultaneous cross-linking of SAgs to both MHC class II molecules on the surface of antigen presenting cells (APC) and T cell receptors at the surface of CD+4 and CD+8 T cells (Abe *et al*., 1991; Akatsuka *et al*., 1994; Li *et al*., 1999; Baker and Acharya, 2004; Petersson *et al*., 2004).

### Interaction with the T cell receptor

As mentioned above, SAg binding to TcR requires an interaction with residues found in the variable (V) region of the β chain, known as the Vβ elements. The other four variable elements of the TcR (e.g., Dβ, Jβ, Vα, and Jα) have little or no effect on TcR-SAg interaction except in rare cases (see below). The human TcR repertoire comprises about 25 different patterns of Vβ elements, known as Vβ families (Tomai *et al*., 1992; Champagne *et al*., 1993; Norrby-Teglund *et al*., 1994; Miyoshi-Akimaya *et al*., 2003; Omoe *et al*., 2004). Each SAg recognizes a specific subset of Vβ families which serves as a characteristic "signature" for an individual SAg (Kotb, 1997; Llewelyn and Cohen, 2002). For example, *S. pyogenes* pyrogenic exotoxin A (scarlet fever/erythrogenic toxin) triggers the activation and expansion of human T cells bearing the Vβ 2, 12, 14, 15 motifs while the pyrogenic exotoxin C recognizes theVβ 1, 2, 5.1, 10 motifs. In contrast, the staphylococcal TSST-1 molecule binds only Vβ 2 motifs (see Alouf and Müller-Alouf, 2003).

The percentage of responding T cells is dictated by the number of Vβ families that interact with a given SAg, as well as by the frequency of expression of SAg-specific Vβ families in an individual's repertoire. As a consequence, SAgs stimulate 5 to 30% of the human T cell repertoire at nano- to picogram concentrations, versus conventional antigen epitopes, which activate and expand less than 0.01% of T cells (Kappler *et al*., 1989; Krakauer 1999; Krakauer and Stiles, 2003).

Certain SAgs (e. g., staphylococcal enterotoxin A and TSST-1) bind to variable elements of the γδ chain of T lymphocytes bearing the appropriate γδ chains (Rust *et al*., 1990; Arvand *et al*., 1995; Morita *et al*., 2001). Interestingly, the staphylococcal enterotoxin H was shown to bind to variable elements of the α chain of TcR (Petersson *et al*., 2003).

### Interaction with MHC class II molecules

SAgs bind to conserved elements of MHC class II molecules with high affinity. Each SAg binds preferentially to distinct alleles of specific MHC isotypes, suggesting differents sites or modes of contact with MHC class II molecules (Choi *et al*., 1989; Scholl *et al*., 1989; Marrack and Kappler, 1990; Imanishi *et al*., 1992; Krakauer, 1999). For example, most staphylococcal enterotoxins bind HLA-DR, whereas many streptococcal pyrogenic exotoxins bind better to HLA-DQ. Differences between different HLA-DR and -DQ alleles might lead to differences between individual susceptibility to particular SAgs (Ulrich *et al*., 1995). Recently, Llwelyn *et al*. (2004) demonstrated that the binding of streptococcal and staphylococcal SAgs to HLA class II is influenced by allelic differences in class II molecules. For the streptococcal pyrogenic exotoxin A, class II binding is dependent upon DQ alpha-chain polymorphisms, such that HLA-DQA1*01 alpha-chains possess greater binding than DQA1*03/05 alpha-chains. The functional implications of differential binding on T cell activation were investigated in various experimental systems using human T cells and murine Vβ 8.2 transgenic cells as responders. Quantitative and qualitative differences resulting from differential HLA-DQ binding were found, as well as changes in T cell proliferation, cytokine production, and Vβ specific changes in T cell repertoire that have hitherto been regarded as a

defining feature of an individual SAg. These observations reveal a mechanism for the different outcomes seen following infection with SAg-producing bacteria. For detailed studies or reviews of SAg interactions with MHC class II molecules and TcR, see Kline and Collins, 1997; McKormick *et al*., 2001; Krakauer and Stiles, 2003; and Petersson *et al*., 2004.

## Structural aspects of SAgs with their receptors

Over the last ten years, X-ray crystallographic determinations of the three-dimensional structures for a substantial number of staphylococcal and streptococcal SAgs, which includes complexes formed with TcR as well as MHC class II molecules, have provided a great deal of information regarding the intricate interactions between these effectors and their receptors (Li *et al*., 1998; Sundberg *et al*., 2002; Baker and Acharya, 2004; Baker *et al*., 2004; Petersson *et al*., 2004; and Chapter 53 of this volume). Certain SAgs were found to possess one or two zinc binding sites, which may have implications in binding to MHC class II molecules. Overall, these studies have shown that despite their common molecular architecture and functional similarities, each SAg may have adopted subtly different ways to cross-link TcR and MHC class II molecules through the use of various structural regions within each SAg.

## Physiological and pathophysiological consequences of SAg interaction with immune system cells

### In vitro *investigations*

As a result of SAg-induced T cell proliferation, a wide array of pro-inflammatory, anti-inflammatory, and regulatory cytokines are massively released *in vitro* by stimulated cells with streptococcal and staphylococcal SAgs. These cytokines are namely comprised of monocyte-derived interleukins IL-1α and β, IL-8 and TNF α-molecules, as well as IL-2, TNF-β, and IFN-γ produced by type 1 helper T lymphocytes (Th1). Besides these pro-inflammatory cytokines, the anti-inflammatory cytokines, IL-4 and IL-10 derived from type 2 helper T lymphocytes (Th2), and the monocyte-derived soluble IL-1 receptor antagonist, are also released following SAg stimulation (Hackett and Stevens, 1993; König *et al*., 1994; Akatsuka et.*al*., 1994; Rink *et al*., 1996; Müller-Alouf *et al*., 1994, 1996, 1997, 2001b; Kotb *et al*., 1997; Krakauer, 1999). Moreover, IL-5, IL-12-, IL-13, and granulocyte-monocyte stimulating factors were also elicited *in vitro* (Müller-Alouf *et al*., 1997). These processes take place through a myriad of up-regulated and down-regulated immunological networks, transduction signals, and the cooperation of adhesion molecules on target cells (see Florkin and Aaldering, 1997; Krakauer, 1999), which ultimately lead to a cascade of events including further release (besides cytokines) of other pharmacologically active products that particularly involve the arachidonic acid/lipid pathways (Hensler *et al*., 1993).

The superantigen-induced up-regulation of cutaneous lymphocyte-associated antigen (CLA) may possibly contribute to the pathogenesis of streptococcal or staphylococcl superantigen-induced skin inflammation (Leung *et al*., 1995c; Zollner *et al*., 1996; Müller-Alouf *et al*., 2001b).

### *Clinical investigations*

*In vivo* pathological overproduction of cytokines and other immunomodulators, particularly those involving the inflammatory cytokine cascade, can be quite harmful. This process may overwhelm the host regulatory network, corrupt immunity (Llwelyn and Cohen, 1992), and cause shock that includes hypotension, tissue damage, and multiorgan failure, which may result in disease and even death (Stevens, 1992; Uchiyama *et al*., 1994; Ulrich *et al*., 1995; Schafer and Sheil, 1995; Leung *et al*., 1995a; Florquin and Aaldering, 1997; Norgren and Eriksson, 1997; Kotb, 1997; Bernal *et al*., 1999; McCormick *et al*., 2001b; Kikuchi *et al*. 2003; Bisno *et al*., 2003). These contentions are supported by a considerable body of experimental (animal models) and clinical evidence. Several investigations revealed the presence of high levels of various cytokines (TNF-α, TNF-β, Il-1α, Il-6, Il-8, IFN-γ) in the acute-phase plasma and/or in the cerebrospinal fluid of patients with severe infections by SAg-producing group A streptococci, particularly in streptococcal toxic shock syndrome (Hackett and Stevens, 1993; Nadal *et al*., 1993; Norrby-Teglund *et al*., 1995; Sriskandan *et al*., 1996). Host variation in cytokine responses to superantigens was shown to determine the severity of invasive group A streptococci (Norrby-Teglund *et al*. 2000, 2001). The detection of streptococcal SAgs in acute-phase serum samples from patients with severe streptococcal diseases has been also reported (Sriskandan *et al*., 1996; Proft *et al*., 2003b).

Tissue biopsy samples from patients with severe invasive streptococcal infections revealed increased expression of IL-1, TNF-β, IFN-γ, and CLA at the local site of infection (Leung *et al*., 1995c; Norrby-Teglund *et al*., 2001).

Other direct evidence for the role of SAgs in disease was also derived from studies that examined specific changes in the TcR Vβ repertoire of patients (Vβ expansion or depletion) during acute illness (Kotb, 1997). For example, flow cytometric analysis of peripheral blood

mononuclear cells from neonates with staphylococcal toxic shock syndrome (TSS) showed specific expansion of TcR Vβ2 positive T lymphocytes (Matsuda *et al*., 2003), thus suggesting *in vivo* stimulation of these cells by the staphylococcal SAg TSST-1. Similar selective expansion of T cells expressing Vβ2 motifs in TSS was earlier reported by Choi *et al*. (1990). Michie *et al*. (1994) reported that lymphocytes collected from two patients with streptococcal toxic shock syndrome (STSS) during the acute phase of the illness possessed: (i) a marked decrease in CD4+ and CD45 RA+(naive) T lymphocytes expressing the Vβ2 chain and (ii) an increase in cells expressing CD+8 and CD45 RO markers. A depletion of Vβ1, Vβ5.1, and Vβ12 patterns was observed in patients with severe streptococcal infections (Watanabe-Ohishi *et al*., 1995).

## IMMUNOPATHOPHYSIOLOGICAL PROPERTIES OF SAGS

Streptococcal and staphylococcal SAgs exhibit a remarkable spectrum of biological and pharmacological activities. These activities concern: (i) the pyrogenic effects elicited by those toxins as a consequence of released IL-1 and TNF-α, and their action upon the hypothalamus; (ii) reticuloendothelial system blockade and enhancement of host susceptibility to lethal shock by endotoxin; (iii) immunosuppression of humoral and cell-mediated responses, deletion of T-cell repertoire, anergy as well as apoptosis of lymphocytes, and (iv) autoimmune disorders (White *et al*., 1989; Marrack and Kappler, 1990; Leung *et al*., 1995a, b; Mahlknecht *et al*., 1996; Florkin and Aaldering, 1997; Norgren and Eriksson, 1997; Monday and Bohach, 1999; Macphail, 1999; Alouf *et al*., 1999; Llewelyn and Cohen, 2002; Mietke *et al*., 1996; Takahashi *et al*., 2001; Krakauer and Stiles, 2003).

## REPERTOIRE OF BACTERIAL SUPERANTIGENS

To date the continually expanding literature describes at least 40 bacterial SAgs produced by Gram-positive bacteria like *Staphylococcus aureus, Streptococcus pyogenes* (and other species), as well as those produced by Gram-negative bacteria such as *Yersinia pseudotuberculosis* and the wall-less *Mycoplasma arthritidis* (see Cole *et al*., 1981, 1991, 1996, 1997; Hodstev *et al*., 1998; Sawitzke *et al*., 2000; Mu *et al*., 2001; Shio *et al*., 2004). It is to be noted that according to Krakauer *et al*. (1997) *C. perfringens* enterotoxin is not a superantigen, which is in contrast to an earlier report by Bowness *et al*. (1992). The superantigenicity of *S. aureus* exfoliatins A and B has been reported (Zollner *et al*., 1996; Monday *et al*., 1999). However, other data indicate that these toxins are not SAgs (Plano *et al*., 2000).

## STAPHYLOCOCCAL SUPERANTIGENS

The repertoire of *S. aureus* SAgs (Lina *et al*. 2004) comprises at least 26 members (including natural molecular variants): (i) the classical staphylococcal enterotoxins (SEs) A, B, C (C1, C2, C3 variants, C ovine, C bovine), D, E and the recently identified enterotoxins G, H, I, J, K, L, M, N, O, P, Q, R (Monday and Bohach, 1999; Jarraud *et al*., 1999; Orwin *et al*., 2001, 2003; McCormick *et al*., 2001a, b; Omoe *et al*., 2004). All SEs are single-chain globular proteins with a molecular mass ranging from 25 to 28k Da (Monday and Bohach, 1999; Alouf and Müller-Alouf 2003; Uchiyama in this volume); (ii) the toxic shock syndrome toxin-1 (TSST-1) (Deresiewicz *et al*., 1994). As its name suggests, TSST-1 discovered in 1981 is the key agent of toxic shock syndrome (TSS). TSST-1 (formerly designated enterotoxin F; see Monday and Bohach, 1999) is a protein consisting of 194 amino acids (22,490 Da), which lacks a disulfide loop and does not induce emesis. An ovine variant of TSST-1 (TSST-O) produced by a mastitis-isolate of *S. aureus* differs from TSST-1 at seven residues (Deresiewicz *et al*., 1994). Both SEs and TSST-1 are relatively heat-stable and resistant to inactivation by proteases. It is worth mentioning that each staphylococcal (and streptococcal) strain generally produces one or more SAgs.

## STREPTOCOCCAL SUPERANTIGENS

A great number of SAgs have been discovered from several species of the *Streptococcus* genus: *Streptococcus pyogenes* (Group A streptococci) (Bisno *et al*., 2003), *Streptococcus equi* (Group C streptococci) (Artiushin *et al*., 2002), Group C and G *Streptococcus dysgalactiae subspecies equisimilis* (Sachse *et al*., 2002; Miyoshi *et al*, 2003; Igwe *et al*., 2003).

### Group A streptococci

To date, the repertoire of *S. pyogenes* SAgs includes eleven structurally and functionally related single-chain proteins with molecular weights ranging from about 23 to 27 kDa (Alouf and Müller-Alouf, 2003,

2004; Petersson *et al*., 2004). Most of these proteins have been purified to homogeneity and include: (i) the classical erythrogenic (scarlet fever) toxins A and C, also designated streptococcal pyrogenic exotoxins A and C (SPE A, SPE C) identified in 1924 and 1960 respectively (see Alouf, 1980; Alouf *et al*., 1999; Norgren and Eriksson, 1997; Alouf and Müller-Alouf, 2003). SPE A (25787 Da) and SPE C (24354 Da) are respectively encoded by bacteriophage genes *speA* and *speC;* and (ii) a series of recently discovered mitogenic exoproteins, namely streptococcal superantigen (SSA) (Mollick *et al*., 1993), SMEZ (for streptococcal mitogenic exotoxin Z) isolated by Kamezawa *et al*. (1997), SPE G, SPE H, SPE I, SPE J (Proft et al., 1999, 2000; McCormick *et al*., 2001a), SPE L, and SPE M (Smoot *et al*., 2002b; Proft *et al*., 2003a).

The occurrence of some of these exotoxins discovered over the past twelve years was initially inferred by genome mapping of *S. pyogenes* serotypes M1, M3, and M18 (Ferretti *et al*., 2001; Beres *et al*., 2002; Smoot *et al*., 2002a). The respective genes were further transcribed and the superantigenicity as well as lethality of corresponding SAg proteins established experimentally (Smoot *et al*., 2002b). Serologic studies showed the presence of antibodies against these SAgs in patients (Proft *et al*., 2003a).

## Genetic determinants of *S. pyogenes*

All known streptococcal SAgs with the exception of SMEZ, SPE G, and SPE J are encoded by bacteriophage genes integrated into the bacterial chromosome (Ferretti *et al*., 2001; Proft *et al*., 2003 b). Four naturally occurring *speA* alleles have been found in strains recovered from patients with severe invasive diseases. Three of these, *speA1*, *speA2*, and *speA3,* encode toxins differing by just a single amino acid. The toxin encoded by *speA4* was 9% divergent from the others with 26 amino acid changes. Strains expressing *speA2* and *speA3* have caused the majority of STSS episodes in the past twelve years, suggesting that the gene products SPE A2 and SPE A3 may be the more bioactive forms of the toxin (Alouf and Müller-Alouf, 2003). This may be due to higher affinity for the HLA-DQ molecule. Four and 24 different alleles of the *spe*C and *smez* genes have been identified, respectively (Proft *et al*., 1999; Arcus *et al*., 2000). Detailed studies on SMEZ-2 (Proft *et al*., 2000) and SMEZ-16 (Müller-Alouf *et al*., 2001b) proteins showed that they are the most potent SAgs (in terms of mitogenic activity) discovered thus far.

## Molecular relatedness

Determination of the nucleotide sequences from the eleven streptococcal SAgs identified to date has revealed various degrees of structural relatedness at both the amino acid sequence and three-dimensional levels. These SAgs share important structural homologies with various *S. aureus* enterotoxin SAgs. Interestingly, certain streptococcal SAgs are more similar to some staphylococcal SAgs than to other streptococcal SAgs. The genomic and structural relatedness suggests that these SAgs share a common ancestor (Arcus *et al*., 2000; Sundberg *et al*., 2002), and these issues are discussed in detail within the ensuing chapters.

## Other streptococcal superantigens

The identification of *spe M, ssa, and smeZ* genes from invasive strains of group C and G *Streptococcus dysgalactiae subspecies equisimilis* isolated from humans has been reported by Igwe *et al*. (2003). The identification of a *spe G* gene in human isolates of *S.dysgalactiae* group C and G was also reported by Sachse *et al*. (2002). A novel SAg produced by *S. dysgalactiae* designated SDM (for *S. dysgalactiae-derived* mitogen) was isolated by Miyoshi-Akiyama (2003). The SDM molecule (212 amino acids) is about 30% homologous with other SAgs at the amino acid sequence level. Phylogenetic analysis indicated that SDM belongs to a family distinct from the established SAgs.

Artiushin *et al*. (2002) identified two novel SAgs, SePE-H (27.5 kDa) and SePE-I (29.5kDa), from *Streptococcus equi*, which cause strangles, an inflammatory disease of the upper respiratory tract and associated lymph nodes of equine species. The two SAgs are respectively related to *S. pyogenes*, SPE H and SPE I, but share only 32% overall homology.

# CONCLUSION

The wealth of information on bacterial SAgs obtained during the past fifteen years has provided a great deal of knowledge regarding the molecular structure, the genetic aspects, and the interaction of these fascinating molecules with the immune system. The key role of SAgs in the pathogenesis of various acute, chronic, and certain autoimmune diseases has afforded new insights into elucidating pathophysiological effects of these molecules. Many questions remain unanswered as yet. New achievements will certainly emerge in the coming years, such as the design and use of novel therapeutical strategies (drugs and vaccines) in the management of SAgs-induced diseases, prophylaxis of susceptible populations against SAg-producing antibiotic resistant (e.g., vancomycin) *S. aureus*, and SAgs targeting toward tumor cells (Kotb, 1999; Macphail, 1999; Krakauer and Stiles, 2003; Arad *et al*., 2004; Petersson *et al*., 2004; Baker and Acharya, 2004).

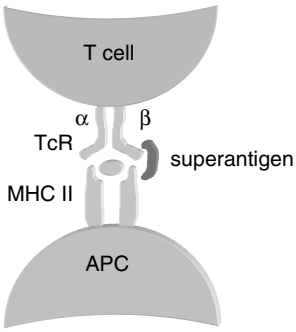



**FIGURE 49.1** Representation of T cell activation by a conventional peptide antigen (Ag) or by a superantigenic toxin (SAg).
TcR: T cell receptor ;
APC: antigen-presenting cell ;
MHC II: major histocompatibility class II complex molecule.

## ACKNOWLEDGMENTS

Christophe Carnoye is gratefully acknowledged for help in generating Figure 49.1. We thank Brad Stiles for helpful advices and critical reading of the manuscript.

## REFERENCES

Abe, J., Forrester, J., Nakahara, T., Lafferty, J.A., Kotzin, B.L. and Leung, D.Y.M., (1991). Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B. *J. Immunol*. **146**, 3747–3750.

Akatsuka, H., Imanishi, K., Inada, K., Yamashita, T., Yoshida, M. and Uchiyama, T. (1994). Production of tumor necrosis factors by human T cells stimulated by a superantigen, toxic shock syndrome toxin-1. *Clin. Exp. Immunol*. **96,** 422–426.

Alouf, J.E. (1980). Streptococcal toxins. Streptolysin O, streptolysin S, erythrogenic toxins. *Pharmacol. Therap*. **11,** 661–717.

Alouf, J.E., Müller-Alouf, H. and Köhler, W. (1999). Superantigenic *Streptococcus pyogenes* erythrogenic pyrogenic exotoxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J. H. Freer), pp. 567–568. Academic Press, London.

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *Int. J. Med. Microbiol*. **292,** 429–440.

Alouf, J.E. and Müller-Alouf, H. (2004). Streptococcus group A. In: *Encyclopedia of Diagnostic Genomics and Proteomics* (pp.1–5), Marcel Dekker Inc., New York.

Arad, G., Hillman, D., Levy, R. and Kaempfer, R. (2004). Broad-spectrum immunity against superantigens is elicited in mice protected from lethal shock by a superantigen antagonist peptide. *Immunol Lett*. **91,** 141–1455.

Arcus, V.L., Proft, T., Sigrell, J.A., Baker, H.M., Fraser, J.D. and Baker, E.N. (2000) Conservation structures of two new superantigens from *Streptococcus pyogenes. J. Mol. Biol*. **299,** 157–168.

Artiushin, S.C., Timoney, J.F., Sheoran, A.S. and Muthupalani, S.K. (2002). Characterization and immunogenicity of pyrogenic mitogens SePE-H and SePE-I of *Streptococcus equi. Microb. Pathogen*. **32,** 71–85.

Arvand, M., Schneider, T., Jahn, J.U. and Hahn, H. (1996). Streptococcal toxic shock syndrome associated with marked γδ T cell expansion: case report. *Clin. Infect. Dis*. **22,** 362–365.

Baker, M.D. and Acharya, K.R. (2004). Superantigens: structure-function relationships. *Int J.Med Microbiol*. **293,** 529–537.

Baker,. H. M., Proft, T., Webb, P.D., Arcus, V.L., Fraser, J.D. and Baker, E.N. (2004). Crystallographic and mutational data show that the streptococcal pyrogenic exotoxin J (SPE-J) can use a common binding surface for T cell receptor binding and dimerization. *J. Biol. Chem*. **279,** 38571–38576.

Beres, S.B., Sylva, G.L., Barbian, K.D., Lei, B., Hoff, J.S., Mammarella, N.D., Liu, M.Y., Smoot, J.C., Porcella, S.F., Parkins, L.D., Campbell, D.S., Smith, T.M., McCormick, J.K., Leung, D.Y., Schlievert, P.M. and Musser, J.M. (2002). Genome sequence of a serotype M3 strain of group A streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc. Natl. Acad. Sci. USA*. **99** 10078–10083.

Bernal, A., Proft, T., Fraser, J.D. and Posnett, D.N. (1999). Superantigens in human disease. *J. Clin. Immunol*. **19,** 149–157.

Bisno, A.L., Brito, M.O. and Collins, M.O. (2003). Molecular basis of group A streptococcal virulence. *The Lancet Infect. Dis*. **3,** 191–200.

Bowness, P., Moss, P.A.H., Tranter, H., Bell, J.I. and McMichael, J. (1992). *Clostridium perfringens* enterotoxin is a superantigen reactive with human T cell receptors Vβ 6.9 an Vβ 22. *J. Exp. Med*. **176,** 893–896.

Carlsson, R., Fischer, H. and Sjögren, H.O. (1988). Binding of staphylococcal enterotoxin A to accessory cells is a requirement for its ability to activate human T cells. *J. Immunol*. **140,** 2484–2488.

Cavaillon, J.-M., Rivière, Y., Svab, J., Montagnier, L. and Alouf, J.E. (1982). Induction of interferon by *Streptococcus pyogenes* extracellular products. *Immunol. Lett*. **5,** 323–6.

Champagne, E., Huchenq, A., Sevin, J., Casteran, N. and Rubin, B. (1993). An alternative method for T cell repertoire analysis: clustering of human V-beta subfamilies selected in response to straphylococcal enterotoxins B and E. *Mol. Immunol*. **30,** 877–886.

Choi,Y. Kotzin, B., Hernon,L., Callahan, J., Marrak, P. and Kappler, K. (1989). Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA* **86,** 8941–8945.

Choi, Y.W., Herman, A. DiGusto, D., Wade, T., Marrack, P. and Kapler, J. (1990). Residues of the variable region of the T cell receptor beta-chain that interacts with *S. aureus* toxin superantigens. *Nature* **346,** 471–473.

Cole, B.C., Daynes, R.A. and Ward, J.R. (1981). Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis*. I. Transformation is associated with an H-2-linked gene that maps to the I-E/I-C subregion. *J. Immunol*. **127,** 1931–1936.

Cole, B.C. and Atkin, C.A. (1991). The *Mycoplasma arthritidis* T cell mitogen, MAM: a model superantigen. *Immunol. Today* **12,** 271–276.

Cole, B.C., Knudston, K.L., Oliphant, A., Sawitzke, A.D., Pole, A., Manohar, M., Benson L.S., Ahmed, E. and Atkin, C.L. (1996). The sequence of the *Mycoplasma arthritidis* superantigen, MAM: identification of functional domains and comparison with microbial superantigens and plant lectin mitogens. *J. Exp. Med*. **183,** 1105–1110.

Cole, B.C., Sawitzke, A.D., Ahmed, E.A., Atkin, C.L. and David, C.S. (1997). Allelic polymorphisms at the H-2A and HLA-DQ loci influence the response of murine lymphocytes to the *Mycoplasma arthritidis* superantigen MAM. *Infect. Immun*. **65,** 4190–4198.

Conrad. B., Weissmahr, R.N., Boni, J., Arcari, R., Schupbach, J. and Mach, B. (1997). A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. *Cell* **90,** 303–313.

Dellabona, P., Peccoud, J., Kappler, J., Marrack, P., Benoist, C. and Mathis, D. (1990). Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* **62,** 1115–1121.

Deresiewicz, R.L., Woo, J.H., Chan, M., Finberg, R.W. and Kasper, D.L. (1994). Mutations affecting the activity of toxic shock syndrome toxin. *Biochemistry* **33,** 12844–12851.

Fast, D.J., Schlievert, P.M. and Nelson, R.D. (1989). Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. *Infect. Immun.* **57,** 291–294.

Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X. Clifton, S.W., Roe, B.A. and McLaughlin, R. (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes. Proc. Natl. Acad. Sci. USA* **98,** 4658–4663.

Fischer, H., Dohlsten, M., Andersson, U., Hedlund, G., Ericsson, P., Hansson, J. and Sjögren H.O. (1990). Production of TNF-α and TNF-β by staphylococcal enterotoxin A activated human T cells. *J. Immunol*. **144,** 4663–4668.

Fleischer, B. and Schrezenmeier, H. (1988). T cell stimulation by staphylococcal enterotoxins. *J. Exp. Med*. **167,** 1697–1707.

Fleischer, B., Schrezenmeier, H. and Conrad, P. (1989). T lymphocyte activation by staphylococcal enterotoxins: role of class II molecules and T cell surface structures. *Cell. Immunol*. **120,** 92–101.

Florquin, S. and Aaldering, L. (1997) Superantigens: a tool to gain new insight into cellular immunity. *Res. Immunol*. **148,** 373–386.

Fraser, J. D. (1989). High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. *Nature* **339,** 221–23.

Fraser, J., Arcus, V., Kong, P., Baker, E. and Proft, T. (2000). Superantigens—powerful modifiers of the immune system. *Mol. Med. Today* **6,** 125–132.

Hackett, S.P. and Stevens, D.L. (1993) Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor β synthesis. *J. Infect. Dis*. **168,** 232–235.

Hensler, T., Köller, M., Geoffroy, C., Alouf, J.E. and König, W. (1993). *Staphylococcus aureus* toxic shock syndrome toxin 1 and *Streptococcus pyogenes* erythrogenic toxin A modulate inflammatory mediator release from human neutrophils. *Infect. Immun*. **61,** 1055–1061.

Hodstev, A.S., Choi, Y., Spanoupoulo, E. and Posnett, D.N. (1998) *Mycoplasma* superantigen is a CDR3-dependent ligand for the T cell antigen receptor. *J. Exp. Med*. **187,** 319–327.

Huber, B.T., Hsu, P-N. and Sulkowski, N. (1996). Virus-encoded superantigens. *Microbiol. Rev*. **60,** 473–483.

Igwe, E.I., Shewmaker, P.L., Facklam, R.R., Farley, M.M., van Beneden, C. and Beall, B. (2003). Identification of superantigen genes *speM, ssa*, and *smeZ* in invasive strains of beta-hemolytic group C and G streptococci recovered from humans. *FEMS Microbiol. Lett*. **229,** 259–264.

Imanishi, K., Igarashi, H. and Uchiyama, T. (1992). Relative abilities of distinct isotypes of human major histocompatibility complex class II molecules to bind streptococcal pyrogenic exotoxins types A and B. *Infect. Immun*. **60,** 5025–5029.

Jarraud, S., Cozon, G., Vandenesch, F., Bes, M., Etienne, J. and Lina, G. (1999). Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J. Clin. Microbiol*. **37,** 2446–2449.

Jupin, C., Anderson, S., Damais, C., Alouf, J.E and Parant, M. (1988). Toxic shock syndrome toxin 1 as an inducer of human tumor necrosis factors and gamma interferon. *J. Exp. Med*. **167,** 752–761.

Kamezawa, Y., Nakahara, T., Nakano, S., Abe, Y. and Nozaki-Renard, J. (1997). Streptococcal mitogenic exotoxin Z, a novel acidic superantigenic toxin produced by a T1 strain of *Streptococcus pyogenes. Infect. Immun*. **65,** 3828–3833.

Kappler, J., Kotzin, B., Herron, L., Gelfand, E., Bigler, R., Boylston, A., Carrel, S., Posnett, D., Choi,Y. and Marrack, P. (1989). Vβ specific stimulation of human T cells by staphylococcal toxins. *Science* **244,** 811–813.

Kikuchi, K., Takahashi, N., Piao, C., Totsuka, K., Nishida, H. and Uchiyama, T. (2003). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* strains causing neonatal toxic shock syndrome-like exanthematous disease in neonatal and perinatal wards. *J. Clin. Microbiol*. **41,** 3001–3006.

Kline, J.B. and Collins, C.M. (1997). Analysis of the interaction between the bacterial superantigen streptococcal pyrogenic exotoxin A (SPEA) and the human T cell receptor. *Mol. Microbiol*. **24,** 197–202.

Kotb, M. (1997). Superantigens in human diseases. *Clin. Microbiol. Newslett*. **19,** 145–152.

König, B., Köller, M., Prévost, G., Piémont, Y., Geoffroy, C., Alouf, J.E., Schreiner, A. and König, W. (1994). Activation of human effector cells by different bacterial toxins (leukocidin, alveolysin, erythrogenic toxin A): generation of interleukin-8. *Infect. Immun*. **62,** 4831–4837.

Krakauer, T., Fleischer, B.H., Stevens, D.L., McClane, B. and Stiles, B.G. (1997). *Clostridium perfringens* enterotoxin lacks superantigenic activity but induces an interleukin-6 response from human peripheral blood mononuclear cells. *Infect. Immun*. **65,** 3485–3488.

Krakauer, T. (1999). Immune response to staphylococcal superantigens. *Immunol. Res*. **20,** 163–173.

Krakauer, T. and Stiles, B.G. (2003). Staphylococcal enterotoxins, toxic shock syndrome toxin-1, and streptococcal pyrogenic exotoxins: some basic biology of bacterial superantigens. *Rec. Res. Devel. Infection & Immunity* **1,** 1–27.

Leung, D.Y.M., Meissner, H.C., Fulton, D.R., Quimby, F. and Schlievert, P.M. (1995a). Superantigens in Kawasaki syndrome. *Clin. Immunol. Immunopathol*. **77,** 119–126.

Leung, D.Y., Travers, J.B., Giorno, R., Norris, D.A., Skinner, R., Aelion, J., Xaremi, L.V., Kim, M.H., Trumble, A.E., Kotb, M. and Schlievert, P.M. (1995b). Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. *J. Clin. Invest*. **96,** 2106–2112.

Leung, D.Y.M. Gately, M., Trumble, A., Ferguson-Darnell, B., Schlievert, P.M. and Pickler, L (1995c). Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen via stimulation of IL-12. *J. Exp. Med*. **181,** 747–753.

Leung, D.Y., Huber, B.T. and Schlievert, P.M. (1997 eds). *Superantigens: Molecular Biology, Immunology, and Relevance to Human Disease*. Marcel Dekker, New York.

Li, H., Llera, A., Malchiodi, E.L. and Mariuzza, R.A. (1999). The structural basis of T cell activation by superantigens. *Annu. Rev. Immunol*. **17,** 435–466.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. and Mariuzza, R. (2004). International Nomenclature Committee for staphylococcal superantigens. Standard nomenclature for the superantigens expressed by *Staphylococcus. J. Infect. Dis*. **189,** 2334–2336.

Llwelyn, M. and Cohen, J. (2002). Superantigens: microbial agents that corrupt immunity. *Lancet Infect. Dis*. **2,** 156–162.

Llewelyn, M., Sriskandan, S., Peakman, M., Ambrozak, R., Douek, D.C., Kwok, W.W., Cohen, J. and Altmann, D.M. (2004). HLA class II polymorphisms determine responses to bacterial superantigens. *J Immunol*. **172,** 1719–1726.

Macphail, S. (1999). Superantigens: mechanisms by which they may induce, exacerbate, and control autoimmune diseases. (1999). *Int. Rev. Immunol*. **18,** 141–180.

Mahlknecht, U., Herter, M., Hoffmann M.K., Niethammer, D. and Dannecker, G.E. (1996). The toxic shock syndrome toxin-1 induces anergy in human T cells *in vivo. Hum. Immunol*. **45,** 42–45.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248,** 705–711.

Matsuda, Y., Kato, H., Yamada, R., Okano, H., Ohta, H., Imanishi, K., Kikuchi, K., Totsuka, K. and Uchiyama, T. (2003). Early and definitive diagnosis of toxic shock syndrome by detection of marked expansion of T cell receptor Vβ2-positive T cells. *Emerg. Infect. Dis*. **9,** 387–389.

McCormick, J.K., Pragman, A.A., Stolpa, J.C., Leung, D.Y. and Schlievert, P.M. (2001a). Functional characterization of streptococcal pyrogenic exotoxin J a novel superantigen. *Infect. Immun*. **69,** 1381–1388.

McCormick, J. K., Yarwood, J.M. and Schlievert, P.M. (2001b). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55,** 77–104.

Michie, C., Scott, A., Cheeseborough, J., Bevereley, P. and Pasvol, G. (1994). Streptococcal toxic-like shock syndrome: evidence of superantigen activity and its effects on T lymphocyte subsets *in vivo. Clin. Exp. Immunol*. **98,** 140–144.

Miethke, T., Vabulas, R., Bittlingermaier, I. R., Heeg, K. and Wagner, H. (1996). Mechanisms of peripheral T cell deletion: anergized T cells are Fas resistant but undergo proliferation associated apoptosis. *Eur. J. Immunol*. **26,** 1459–1467.

Miyoshi-Akoyama, T., Zhao, J., Kato, H., Kikuchi, K., Totsuka, K., Kataoka, Y., Katsumi, M. and Uchiyama, T. (2003). *Streptococcus dysgalactiae*-derived mitogen (SDM), a novel bacterial superantigen: characterization of its biological activity and predicted tertiary structure. *Mol. Microbiol*. **47,** 1589–1599.

Mollick, J., Cook, R. and Rich, R. (1989). Class II MHC molecules are specific receptors for staphylococcal enterotoxin A. *Science*, **244,** 817–819.

Mollick, J.A., Miller, G.G., Musser, J.M., Cook, R.G., Grossman, D. and Rich, R.R. (1993) A novel superantigen isolated from pathogenic strains of *Streptococcus pyogenes* with aminoterminal homology to staphylococcal enterotoxins B and C. *J. Clin. Invest*. **92,** 710–719.

Monday, S.R. and Bohach, G.A. (1999). Properties of *Staphylococcus aureus* enterotoxins and toxic shock syndrome toxin-1. In*: The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), pp. 589–610. Academic Press, London.

Morita, C.T., Li, H., Lamphear, J.G., Rich, R.R., Fraser, J.D., Mariuzza, R.A. and Lee, K. (2001). Superantigen recognition by gamma delta T cells: SEA recognition site for human V gamma 2 T cell receptors. *Immunity* **14,** 331–344.

Mu, H.H., Sawitzke, A.D. and Cole B.C. (2001). Presence of Lps(d) mutation influences cytokine regulation *in vivo* by the *Mycoplasma arthritidis* mitogen superantigen and lethal toxicity in mice infected with *M. arthritidis. Infect. Immun*. **69,** 3837–3844.

Müller-Alouf, H., Alouf, J.E., Gerlach, D., Ozegowski, J.-H., Fitting, C. and Cavaillon, J.M. (1994). Comparative study of cytokine release by human peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes* superantigenic erythrogenic toxins, heat-killed streptococci, and lipopolysaccharide. *Infect. Immun*. **62,** 4915–4921.

Müller-Alouf, H., Alouf, J.E., Gerlach, D., Ozegowski, J.-H., Fitting, C. and Cavaillon, J.M. (1996). Human pro- and anti-inflammatory cytokine pattern induced by *Streptococcus pyogenes* erythrogenic (pyrogenic) exotoxins A and C superantigens. *Infect. Immun*. **64,** 1450–1453.

Müller-Alouf, H., Gerlach, D., Desreumaux, P., Leportier, C., Alouf, J. E. and Capron, M. (1997). Streptococcal pyrogenic exotoxin A (SPE A) superantigen-induced production of hematopoietic cytokines, IL-12 and IL-13, by human peripheral blood mononuclear cells. *Microb. Pathogen*. **23,** 265–272.

Müller-Alouf, H., Carnoy, C., Simonet, M. and Alouf, J.E. (2001a). Superantigen bacterial toxins: state of the art. *Toxicon* **39,** 1691–1701.

Müller-Alouf, H., Proft, T., Zollner, T.M., Gerlach, D., Champagne, E., Desreumaux, P., Fitting, C., Geoffroy-Fauvet, C., Alouf, J.E. and Cavaillon, J.M. (2001b). Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. *Infect. Immun*. **69,** 4141–4145.

Nadal, D., Lauener, R.P., Braegger, C.P., Kaufhold, A., Simma, B., Lütticken, R. and Seger, A. (1993). T cell activation and cytokine release in streptococcal toxic shock-like syndrome. *J. Pediatr*. **122,** 727–729.

Norgren, M. and Eriksson, A. (1997). Streptococcal superantigens and their role in the pathogenesis of severe infections. *J. Toxicol. Toxin Rev*. **16,** 1–32.

Norrby-Teglund, A., Newton, D., Kotb, M., Holm, S.E. and Norgren, M. (1994). Superantigenic properties of the group A streptococcal exotoxin SPE F (MF). *Infect. Immun*. **62,** 5227–5233.

Norrby-Teglund, A., Pauksens, K., Norgren, M. and Holm, S.E. (1995). Correlation between serum TNF α and IL-6 levels and severity of group A streptococcal infections. *Scand J. Infect Dis*. **27,** 125–130.

Norrby-Teglund, A., Chetellier, S., Low, D., McGeer, A., Green, K. and Kotb, M. (2000). Host variation in cytokine responses to superantigens determine the severity of invasive group A streptococcal infection. *Eur. J. Immunol*. **30,** 3247–3255.

Norrby-Teglund, A., Thulin, P., Gan, B.S., Kotb, M., McGeer, A., Andersson, J. and Low, D.E. (2001). Evidence for superantigen involvement in severe group A streptococcal tissue infections. *J. Infect. Dis*. **184,** 853–860.

Omoe, K., Imanishi, K., Hu, D.L., Kato. H., Takahashi-Omoe, H., Nakane, A., Uchiyama, T. and Shinagawa, K. (2004). Biological properties of staphylococcal enterotoxin-like toxin type R. *Infect Immun*. **72,** 3664–3667.

Orwin, P.M., Leung, D.Y., Donahue, H.L., Novick, R.P. and Schlievert, P.M. (2001). Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun*. **69,** 360–366.

Orwin P.M., Fitzgerald, J.R., Leung, D.Y., Gutierrez, J.A., Bohach, G.A. and Schlievert, P.M. (2003). Characterization of *Staphylococcus aureus* enterotoxin L. *Infect. Immun*. **71,** 2916–2919.

Parsonnet, J., Hickman, R.K., Eardley, D.D. and Pier, G.B. (1985). Induction of human interleukin-1 by toxic shock syndrome toxin–1. *J. Inf. Dis*. **151,** 514–522.

Parsonnet, J. and Gillis, Z.A. (1988). Production of tumor necrosis factor by human monocytes in response to toxic shock syndrome toxin–1. *J. Inf. Dis*. **158,** 1026–33.

Petersson, K., Petersson, H., Skartved, N.J., Walse, B. and Forsberg, G. (2003). Staphylococcal enterotoxin H induces V alpha-specific expansion of T cells. *J. Immunol*. **170,** 4148–4154.

Petersson, K., Forsberg, G. and Walse, B. (2004). Interplay between superantigens and immunoreceptors. *Scand. J. Immunol*. **59,** 345–355.

Plano, LR., Gutman, DM., Woischnik, M. and Collins, CM. (2000). Recombinant *Staphylococcus aureus* exfoliative toxins are not bacterial superantigens. *Infect Immun*. **68,** 3048–3052.

Proft, T., Moffat, S.L. and Fraser, J.D. (1999). Identification and characterization of novel superantigens from *Streptococcus pyogenes. J. Exp. Med*. **189,** 89–102.

Proft, T., Moffat, S.L., Weller, K.D., Paterson, A., Martin, D. and Fraser, J.D. (2000). The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. *J. Exp. Med*. **197,** 1765–1767.

Proft, T., Webb, P.D., Handley, V. and Fraser, J.D. (2003a). Two novel superantigens found in both group A and group C streptococcus. *Infect. Immun*. **71,** 1361–1369.

Proft, T., Sriskandan, S., Yang, L. and Fraser, J.D. (2003b). Superantigens and streptococcal toxic shock syndrome. *Emerg. Infect. Dis*. **9,** 1211–1218.

Rink, L., Luhm, J., Koestler, M. and Kirchner, H. (1996). Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J. Interferon Cytokine Res*. **16,** 41–47.

Rust, C.J.J., Verreck, F, Vietor, H. and Koning, F. (1990). Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the Vγ9 region. *Nature* **346,** 572–574.

Sachse, S., Seidel, P., Gerlach, D., Günther, E., Rodel, J., Straube, E. and Schmidt, K.H. (2002). Superantigen-like gene(s) in human pathogenic *Streptococcus dysgalactiae, subspecies equisimilis*: genomic localization of the gene encoding streptococcal pyrogenic exotoxin G [spe(G) dys)]. *FEMS Immunol. Med. Microbiol*. **34,** 159–167.

Sawitzke, A., Joyner, D., Knudson, K., Mu, H.H. and Cole, B. (2000). Anti-MAM antibodies in rheumatic disease: evidence for a MAM-like superantigen in rheumatoid arthritis? *J. Rheumatol*. **27,** 358–364.

Schafer, R. and Sheil, J.M. (1995). Superantigens and their role in infectious diseases. *Adv. Pediatr. Infect. Dis*. **10,** 369–390.

Scholl, P.R., Diez, A., Karr, R., Sekay, J., Trowsdale, J. and Geha, R.S. (1989). Effects of isotypes and allelic polymorphism on the binding of staphylococcal exotoxins to MHC class II molecules. *J. Immunol*. **144,** 226–230.

Shio, M.T., Ribeiro-Dias, F., Timenetsky, J. and Jancar, S. (2004). PAF is involved in the *Mycoplasma arthritidis* superantigen-triggering pathway for iNOS and COX-2 expression in murine peritoneal cells. *Exp. Cell Res*. **298,** 296–304.

Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Ricklefs, S.M., Porcella, S.F., Parkins, L.D., Beres, S.B., Campbell, D.S., Smith, T.V., Zhang, Q., Kapur, V., Daly, J.A., Veasy, L.G. and Musser, J.M. (2002a). Genome sequence and comparative microarray analysis of serotype M18 group A streptococcus strains associated with acute rheumatic fever outbreaks. *Proc. Natl. Acad. Sci. USA*. **99,** 4668–4673.

Smoot, L.M., McCormick, J.K., Smoot, J.C., Hoe, N.P., Strickland, I., Cole, R.L., Barbian, K.D., Earhart, C.A., Ohlendorf, D.H., Veasy, L.G., Hill, H.R., Leung, D.Y., Schlievert, P.M. and Musser, J.M. (2002 b). Characterization of two novel pyrogenic toxin superantigens made by an acute rheumatic fever clone of *Streptococcus pyogenes* associated with multiple disease outbreaks. *Infect. Immun*. **70,** 7095–7104.

Sriskandan, S., Moyes, D. and Cohen, J. (1996). Detection of circulating bacterial superantigen and lymphotoxin–α in patients with streptococcal toxic shock syndrome. *The Lancet* **348,** 1315–1316.

Stauffer, Y., Marguerat, S., Meylan, F., Ucla, C., Sutkowski, N., Huber, B., Pelet, T. and Conrad, B. (2001). Interferon alpha-induced endogenous superantigen: a model linking environment and autoimmunity. *Immunity* **15,** 591–601.

Stevens, D.L. (1992). Invasive group A streptococcus infections. *Clin. Infect. Dis*. **14,** 2–13.

Sundberg, E.J., Li, H., Llera, A.S., McCormick, J.K., Tormo, J., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (2002). Structures of two streptococcal superantigens bound to TCR chains reveal diversity in the architecture of T cell signaling complexes. *Structure* **10,** 687–699.

Sutkowski, N., Conrad, B., Chen, G., Calderon, G. and Huber, B.T. (2004). Epstein-Barr virus latent membrane protein LMP-2A is sufficient for transactivation of the human endogenous retrovirus HERV-K18 superantigen. *J. Virol*. **78,** 7852–7860.

Takahashi, M., Shinohara, F., Takada, H. and Rikiishi, H. (2001). Effects of superantigen and lipopolysaccharide on induction of CD80 through apoptosis of human monocytes. *Infect. Immun*. **69,** 3652–3657.

Tomai, M.A., Schlievert, P.M. and Kotb, M. (1992). Distinct T cell receptor Vβ usage by human T lymphocytes stimulated with the streptococcal pyrogenic exotoxins and pepM5 protein. *Infect. Immun*. **60,** 701–705.

Uchiyama, T., Imanishi, K., Saito, S., Araake, M., Yan, X.J., Fujikawa, H., Igarashi, H., Kato, H., Obata, F., Kashiwagi, N. and Inoko, H. (1989a). Activation of human T cells by toxic shock syndrome toxin-1: the toxin-binding structures expressed on human lymphoid cells acting as accessory cells are HLA class II molecules. *Eur. J. Immunol*. **191,** 1803–1809.

Uchiyama, T., Kamagata,Y., Yan, X.J., Kawachi, H., Fujikawa, H., Igarashi, H. and Okubo, M. (1989b). Relative strength of the mitogenic and interleukin-2 production-inducing activity of staphylococcal exotoxins presumed to be causative exotoxins of toxic shock syndrome toxin-1 and enterotoxins A, B, and C to murine and human T cells. *Clin. Exp. Immunol*. **75,** 239–244.

Uchiyama, T., Yan, X.-J., Imanishi, K. and Yagi, Y. (1994). Bacterial superantigens: mechanism of T cell activation by the superantigens and their role in the pathogenesis of infectious diseases. *Microbiol. Immunol*. **38,** 245–256.

Ulrich, R.G., Bavari, S. and Olson, M.A. (1995). Bacterial superantigens in human diseases: structure, function, and diversity. *Trends Microbiol*. **3,** 463–468.

Watanabe-Ohnishi, R., Low, D.E., McGeer, A., Stevens, D.L., Schlievert, P.M., Newton, D., Schwartz, B. and Kreiswirth, B. *et al*. (1995). Selective depletion of Vβ bearing T cells in patients with severe group A streptococcal infections and streptococcal toxic shock syndrome. *J. Infect. Dis*. **171,** 74–84.

White, J., Herman, A., Pullen, M., Kubo, R., Kappler, J.W. and Marrack, P. (1989). The Vβ specific superantigen staphylococcal enteroxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56,** 27–35.

Wirth, S., Vessaz, A., Krummenacher, C., Baribaud, F., Acha-Orbea, H. and Diggelmann, H. (2000). Regions of mouse mammary tumor virus superantigen involved in interaction with the major histocompatibility complex class II I-A molecule. *J. Virol*. **76,** 1172–1175.

Zollner, T.M., Munk, M.E., Keller, T., Nuber, V., Boehnke, W.H., Kaufmann, S.H.E., Duijvesijn, A.M., Sterry, W. and Kaufmann, R. (1996). The superantigen exfoliative toxin induces cutaneous lymphocyte-associated antigen expression in peripheral human T lymphocytes. *Immunol. Lett*. **49,** 111–116.

CHAPTER

# 50

# Staphylococcal superantigens and the diseases they cause

*Takehiko Uchiyama, Ken'ichi Imanishi, Tohru Miyoshi-Akiyama, and Hidehito Kato*

## INTRODUCTION

Seventeen years have passed since the superantigen (SAG) concept was proposed for several exotoxins from *Staphylococcus aureus* (*S. aureus)*, such as toxic shock syndrome (TSS) toxin-1 (TSST-1) and enterotoxin A (SEA) and B (SEB) (White *et al*., 1989; Kappler *et al*. 1989). The binding structures essential for their SAG activity were also found in the same year to be MHC class II molecules (Fraser *et al* 1989; Herrmann *et al* 1989; Mollik *et al* 1989; Scholl *et al* 1989; and Uchiyama *et al*., 1989). Subsequently, a few other microbial species, such as *Streptococcus pyogenes* (*Str. pyogenes*) (Imanishi *et al*., 1990), mammary tumor virus in mice (Held *et al*., 2004), *Mycoplasma arthritidis* (Cole *et al*., 1991), and *Y. pseudotuberculosis* (*Y. ptbc*.) (Abe *et al* 1993; Uchiyama *et al*., 1993; and Miyoshi-Akiyama *et al*. 1995), were found to produce a handful of SAGs. Recently, staphylococcal and streptococcal species other than *S. aureus* and *S. pyogenes* were also reported to produce SAGs. To date, approximately 30 bacterial SAGs have been identified. A remarkable biological feature of SAGs is their capacity to stimulate T cells in vast numbers in a T cell receptor (TCR) Vβ element-specific manner in direct association with MHC class II molecules on antigen-presenting cells (APCs). It is well known that some staphylococcal enterotoxins cause food poisoning. In addition, the primary involvement of T cell activation by several SAGs, including TSST-1 in the pathogenesis of infectious diseases manifesting acute and systemic clinical symptoms, such as toxic shock syndrome (TSS) (Uchiyama *et al*., 1986, 1987, 1989a, and 1989b), and neonatal TSS-like exanthematous diseases (NTED) (Takahashi *et al*., 1998 and 2000) has been established. Another bacterial SAG is also a key pathogenic factor in *Y. ptbc*. infection (Abe *et al*. 1993; Uchiyama *et al*., 1993). A number of excellent reviews on bacterial SAGs have recently been published (Alouf and Muller-Alouf, 2003; McCormick *et al*., 2001; and Proft and Fraser, 2003).

Herein, we will review progress in research into new staphylococcal SAGs and the infectious diseases they cause, focusing on pathogenic mechanisms and early definitive disease diagnosis.

## EMERGENCE OF STAPHYLOCOCCAL SAGS

Staphylococcal SAGs, the location of SAG genes, and Vβ elements in the TCR β chain expressed in SAG-reactive T cells are presented in Table 50.1. Some staphylococcal enterotoxins were originally identified as enterotoxins because of their emetic activity. The international nomenclature committee on staphylococcal SAGs has proposed that only toxins exhibiting emetic activity after oral administration in a primate model be designated staphylococcal enterotoxins (Lina *et al*., 2004). Other related toxins lacking emetic activity or as yet untested for this activity were recommended to be designated "staphylococcal enterotoxin-like toxin type X (SElX)." In Table 50.1, names of toxins lacking emetic activity or which have not yet been tested for

this activity are listed according to the originally reported names and names arbitrarily revised by our group according to the SEIX nomenclature.

Of approximately 20 staphylococcal SAGs, SEA, SEB, staphylococcal enterotoxins C, D, and E (SEC1, SEC2, and SEC3, SED, SEE), and TSST-1 had already been discovered prior to proposal of the SAG concept. All of these toxins other than TSST-1 were well known to have emetic activity and to cause food poisoning (Bergdoll, 1979). Nucleotide sequences of the genes encoding these toxins were fully elucidated during the 1985–1990 period: SEA (Betley and Mekalanos, 1988), SEB (Ranelli *et al*., 1985), SECs (Bohach and Schlievert, 1985 and 1987; and Hovde *et al*., 1990), SED (Bayles and Iandolo, 1989), SEE (Cough *et al*., 1988), and TSST-1 (Blomster-Hautamaa *et al*., 1986). A group of enterotoxins share in common a disulphide bond, which is not directly involved in the aforementioned activity, but plays a role in maintaining the tertiary structure needed for this activity (Hovde *et al*., 1994). As to TSST-1, two research groups independently isolated a toxin from *S. aureus* strains isolated from patients with TSS and found this toxin to be the cause of TSS in 1981. The toxins were initially called pyrogenic exotoxin (Schlievert *et al*., 1981) or enterotoxin F (Bergdoll *et al*., 1981). Ultimately, however, the toxin was renamed TSST-1 because it was identified as the same molecule based on amino acid profiles and immunological cross-reactivity (Bonventre *et al*., 1983). TSST-1 was found to be produced by all of *S. aureus* strains isolated from TSS patients, and at low rates in isolates from individuals with no history of TSS (Bonventre *et al*. 1983; Cohen *et al*., 1983). In five (17.2%) of 29 TSS patients, sera contained antibodies (Abs) to the toxin, but only four (4.6%) of 87 isolates from other sources contained these antibodies (Bergdoll *et al*., 1981). TSST-1 was recognized as a pyrogen that enhanced susceptibility to lethal shock caused by endotoxin (Schlievert *et al*., 1981). These findings strongly suggested that TSST-1 is the major causative toxin of TSS. A key finding elucidating the pathogenic mechanism of TSS was the potent capacity of TSST-1 to activate T cells, a finding that was independently reported by three groups of investigators in the mid-1980s (Calvano *et al*., 1984; Poindexter and Schlievert, 1985; Uchiyama *et al*., 1986). The SAG activities of staphylococcal toxins described have been reviewed already elsewhere (Uchiyama *et al*., 1994). SEA, SEB, and SEC can function as pathogenic toxins in TSS in addition to TSST-1, because *S. aureus* strains producing these toxins in the absence of TSST-1 have been isolated from TSS patients (Crass and Bergdoll, 1986).

SEG and SEI were purified and their DNA sequences were determined in 1998 (Munson *et al*., 1998). The former exhibited emetic activity and a disulphide bond, while the latter lacked a disulphide bond and exhibited significantly less emetic activity. Both were also found to be produced by strains isolated from TSS patients negative for production of either SEA-SEE or TSST-1 (Jarraud *et al*., 1999), suggesting their involvement in the pathogenesis of TSS. SEG and SEI selectively activate human T cells with TCR expressing Vβ14, and T cells with Vβ5.1 and Vβ7, respectively (Jarraud *et al*., 2001). SEH was purified from a TSST-1 negative strain isolated from a TSS case, and its DNA sequence was determined in 1994 (Ren *et al*., 1994). This toxin showed emetic activity in rhesus monkeys (Su and Wong, 1995) and had the disulphide bond. SEH appears to be unique in that it stimulates T cells in a TCR Vα10-selective, but not TCR Vβ-selective manner (Petersson *et al*., 2003). An SED-encoding plasmid was found to encode a previously unidentified enterotoxin designated SEJ (Zhang *et al*., 1998). Grouping of SEJ into the SAG family may have to be delayed, however, because its SAG activity has not yet been determined. Genes for SEK and SEL were detected in a family of mobile pathogenicity islands of *S. aureus* (Lindsay *et al*., 1998). SEK

**TABLE 50.1** Staphylococcal SAGs and TCR Vβ repertoires of human T cells reactive with them, and gene location of them

| SAGs reported names | Arbitrarily revised names | Human TCR Vβ (Vα) specificity | Gene location |
|---|---|---|---|
| SEA | | 1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, 18 | phage |
| SEB | | 3, 12, 14, 15, 17, 20 | PI |
| $SEC_1$ | | 3, 6.4, 6.9, 12, 13.2, 14, 15, 17, 20 | |
| $SEC_2$ | | 12, 13.2, 14, 15, 17, 20 | PI |
| $SEC_3$ | | 3, 5, 12, 13.1, 13.2 | |
| SEC canine | | 3, 5.2, 12, 13.2, 14, 15, 17, 20 | |
| SED | | 5, 12 | plasmid |
| SEE | | 5.1, 6.3, 6.4, 6.9, 8.1, 18 | not plasmid |
| SEG | | 13.6, 14, 15 | PI |
| SEH | | Vα10 | chromosomal? |
| SEI | | 1, 5.1, 5.2, 5.3, 23 | PI |
| SEJ | SElJ | ND | plasmid |
| SEK | SElK | 5.1, 5.2, 6.7 | PI |
| SEL | SElL | 5.1, 5.2, 6.7, 16, 22 | PI |
| SEM | SElM | 18, 21.3 | PI |
| SEN | SElN | 9 | PI |
| SEO | SElO | 5.1, 7, 22 | PI |
| SElP | | 5.1, 6, 8, 16, 18, 21.3 | phage |
| SEQ | SElQ | 2, 5.1, 5.2, 6.7, 21.3 | PI |
| SElR | | 3, 11, 12, 13.2, 14 | unidentified |
| SEU | SElU | ND | PI |
| TSST-1 | | 2, 4 | PI |

SEA-SEU, staphylococcal enterotoxin A-U; TSST-1, toxic shock syndrome toxin-1; SElJ-SElU, staphylococcal enterotoxin-like toxin type J-U; PI, pathogenicity island

and SEL selectively activate human T cells with TCR expressing Vβ5.1, Vβ5.2, and Vβ6.7, and TCR expressing Vβ5.1, Vβ5.2, Vβ6.7, Vβ16, and Vβ 22, respectively (Orwin *et al*., 2001, 2003). Both molecules lack the disulphide bond. SEL was found to have no emetic activity, while SEK was not examined in this regard. Sequence analysis of *seg-sei* intergenic DNA and flanking regions revealed that open reading frames for new SAGs, SEM, SEN, and SEO, in addition to *seg* and *sei*, were encoded in a tandem orientation (Jarraud *et al*., 2001). Although SEN and SEO were initially designated SEK and SEL, respectively, these toxins were different from the SEL and SEK reported by Orwin *et al*. in 2001 and 2003. The authors renamed their toxins SEN and SEO (Jarraud *et al*., 2001). It was found that the recombinant forms of these SAGs activate human T cells with TCR expressing Vβ21.3 in SEM, Vβ9 in SEN, and Vβ 5.1 and Vβ 7 in SEO. The gene *selp* that codes SElP (staphylococcal enterotoxin-like toxin P) was cloned from an *S. aureus* strain causing food poisoning. Recombinant SElP activates human T cells in a Vβ 5, 6, 8, 16, 18, 21-selective manner (Omoe *et al*., 2005). A gene coding SEQ was cloned from an *S. aureus* strain isolated from TSS patients, and a recombinant SEQ selectively activated human T cells with TCR expressing Vβ 2, Vβ 5.1, Vβ 5.2, Vβ 6.7, and Vβ 21.3 (Orwin *et al*., 2002). SEQ did not show emetic activity and lacked the disulphide bond. A gene coding SElR (staphylococcal enterotoxin-like toxin type R) was cloned from a strain of *S. aureus* that had caused an outbreak of food poisoning, and its recombinant protein was initially designated staphylococcal enterotoxin R (Omoe *et al*., 2003). The molecule, which was later renamed SElR according to the nomenclature committee due to lack of emetic activity testing, selectively activates human T cells with TCR expressing Vβ 3, Vβ 11, Vβ 12, Vβ 13.2, and Vβ 14 (Omoe *et al*., 2004). This toxin contains the disulphide bond. A gene coding SEU (staphylococcal enterotoxin U) was identified within the *egc* cluster that codes SEG, SEI, SEM, SEN, and SEO (Letertre *et al*., 2003). Its SAG and emetic activities have not as yet been examined, however.

A dendrogram of staphylococcal SAGs is presented as Figure 50.1. Apparently, staphylococcal SAGs can be divided into three major monophyletic groups and two minor groups. Group 1 contains the classic SAGs SEA, SED, and SEE, and some newly identified SAGs. Group 2 contains the classic SAGs SEB and SEC, and some newly identified SAGs. Group 3 is composed of only newly recognized SAGs alone. Groups 4 and 5 contained TSST-1 and SEH alone, respectively. Proteins in groups 1, 2, and SEH have the disulphide bond, and emetic activity has been confirmed as being present or absent. Proteins in group 3 and TSST-1 lack the disulphide bond, and activity has been confirmed as being low or absent, or has not yet been tested.

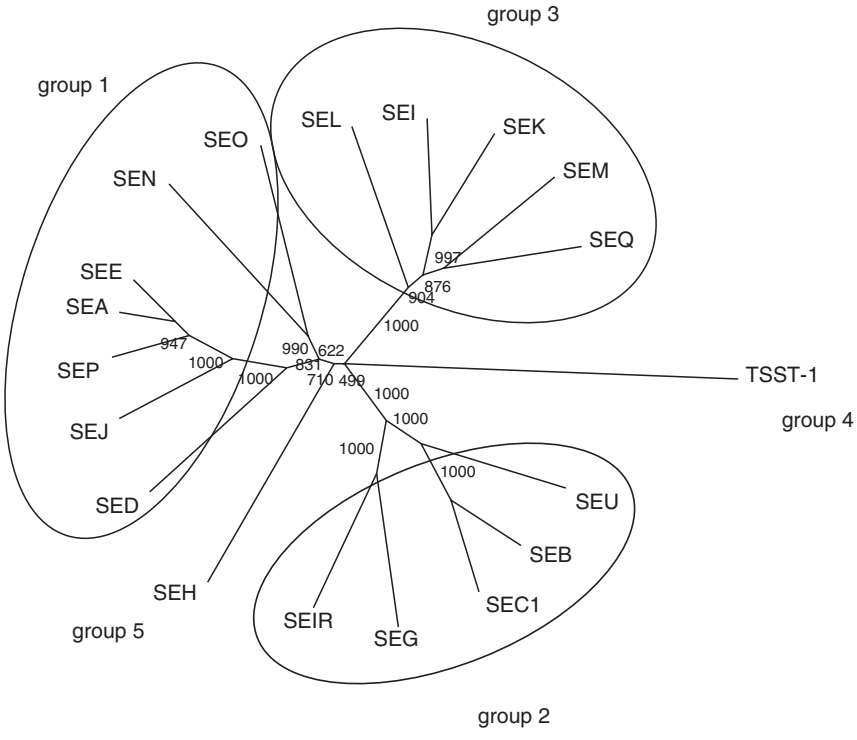



**FIGURE 50.1** Dendrogram of staphylococcal SAGs. The amino acid sequences of staphylococcal SAGs were aligned using the Clustal W program. Evolutionary distances were determined according to the correction of Kimura, and distance values were used to construct a dendrogram by the neighbor-joining method with the Clustal W program. The SAG dendrogram was drawn with the TREEVIEW program. The numbers at the nodes represent the bootstrap values based on 1,000 resampling trials.

Recent analyses have suggested that staphylococcal species other than *S. aureus* produce SAGs. For example, a molecule designated $SEC_{canine}$, which has high sequence identity with SEC2 and SEC3, has been identified from *S. intermedius* (Edwards *et al*., 1997). This molecule exhibited emetic activity and selectively stimulates human T cells with TCR Vβ repertoires similar to those of SEC1.

Recent genome analyses revealed that clusters of genes, 26 genes in total, coding SAG-like proteins, designated staphylococcal enterotoxin-like toxins (SETs), are encoded in the pathogenicity island at chromosomal region RD13 of several organisms (Williams *et al*., 2000; Kuroda *et al*., 2001; and Baba *et al*., 2002), mainly methicillin-resistant *S. aureus* (MRSA) strains. The crystal structure of one member of the SET family, SET3, shows low amino acid sequence homology with TSST-1 (26%), and shares a structure that is conserved in all bacterial SAGs. Several studies have shown, however, that SETs have no capacity to stimulate human T cells (Arcus *et al*., 2002; Kato *et al*., manuscript in preparation). The nomenclature committee recommended renaming members of this protein family the "staphylococcal superantigen-like" (SSL) proteins (Lina *et al*., 2004). It is our current opinion that these "SSL" proteins cannot be grouped into the SAG family.

## SAG ACTIVITY AND T CELL-DEPENDENT PATHOGENIC ACTIVITY OF STAPHYLOCOCCAL TOXINS

Staphylococcal SAGs exhibit potent T cell-stimulating activities that are closely related to the pathogenic mechanisms of TSS and NTED.

### SAG activity

Conventional protein antigens bind to MHC class II molecules inside their antigen-binding groove on the surfaces of APCs, such as macrophages and dendritic cells, after being processed into peptides by these cells, and the resulting complexes of peptides and MHC class II molecules activate T cells in small numbers. T cells use all five variable elements of the TCR, including Vα and Jα elements in the α chain and Vβ, Dβ, and Jβ elements in the β chain, to recognize activating antigenic complexes (we estimate activated cells to be at a level of $10^2$/respective peptide antigen). By comparison, SAGs behave toward T cells like "super antigens." They bind directly to MHC class II molecules outside their antigenic peptide-binding groove on the surfaces of APCs without being processed. Subsequently, the SAG . MHC class II complexes selectively activate virtually all T cells expressing particular Vβ elements in the TCR β chain. Variable elements other than Vβ are not intimately involved in the recognition of SAGs with the exception of SEH. For example, TSST-1 activates at quite low *in vitro* doses (1–10 pg/ml) virtually all human T cells expressing Vβ2 or Vβ4 in the TCR β chain, which account for more than 10% of total T cells, to induce proliferation and the production of various cytokines. SEH is reported to stimulate human T cells in a TCR Vα-selective manner. Human T cells expanded in response to stimulation with SEH, expressing TCR Vα10, while no TCR Vβ-specific expansion was seen (Petersson *et al*., 2003).

### T cell-dependent pathogenic mechanisms of TSS and other SAG-induced diseases

Histopathological findings of fatal TSS cases examined during the late 1970s and the early 1980s in the United States showed aseptic inflammatory lesions in multiple organs and abnormal changes in lymphoid tissues, including lymphopenia or lymphocytosis and erythrophagocytosis by macrophages (Larkin *et al*., 1982; Paris *et al*., 1982), suggesting that TSST-1-induced activation had occurred in the lymphoid tissues of patients. Rabbits receiving continuous administration of TSST-1 via implanted diffusion chambers filled with TSST-1-producing *S. aureus* (Rasheed *et al*., 1985) or osmotic pumps filled with TSST-1 (Parsonnet *et al*., 1987) exhibited histopathological changes similar to those in human TSS cases. TSST-1 is a potent T cell stimulant inducing massive proliferation and production of cytokines, as described previously. Side effects resembling clinical symptoms observed in TSS patients were seen in humans administered large doses of cytokines, which could be induced by TSST-1 (Gutterman *et al*., 1980; Lotze *et al*., 1985). TSST-1 produced no direct cytotoxic effect on cultured cell lines, while orthodox bacterial toxins, such as Shiga toxin and diphtheria toxin, were cytotoxic in such cell cultures. Taking these findings into consideration, it was hypothesized in the late 1980s that lymphokines produced in excess amounts by T cells activated with TSST-1 were primarily involved in the pathogenic mechanism of TSS (Uchiyama *et al*., 1986, 1987, 1989a, 1989b).

Later, T cell activation by TSST-1 was shown to be involved actually in the pathogenic mechanism of TSS. Examinations of T cells in peripheral blood mononuclear cells (PBMs) from patients with TSS by semiquantative PCR showed that massive expansion of $V\beta2^+$ T cells occurred at the acute phase (Choi *et al*., 1990). Recently, we determined the percentage of $V\beta2^+$ T cells using an immunologic method in which PBM T cells

were stained with various fluorescent dye-labeled Abs. It was found that the percentage of Vβ2$^+$ T cells increased beyond the control level in the acute phase in TSS patients (Matsuda *et al*., 2003) and NTED patients (Takahashi *et al*., 1998, 2000), as shown in Figure 50.2, and approached the control levels during recovery. The percentage of Vβ2$^+$CD4$^+$ T cells strongly expressing CD45RO was increased from the control level (5–8%) to approximately 28% in a TSS case and 24% in a NTED case. CD45RO is expressed in recently activated T cells. The results showed that activation of TSST-1-reactive T cells with TSST-1 occured in TSS and NTED patients. Experimental findings using mice also supported the validity of this view. Wasting was seen in normal mice, but not in mice lacking T cells, when mice were repeatedly injected with SEB from the neonatal stage (Marrack *et al*., 1990). Mutated SEB, which retains the ability to bind to MHC class II molecules but lacks T cell-stimulating activity, did not induce wasting.

A question arises as to the involvement of SAG activity in the emetic activity of staphylococcal enterotoxins. Investigations have supported the notion that the emetic activities of SEA and SEB are not mediated via their effects on T cells (Alber *et al*., 1990; Harris and Betley, 1995; Hoffman *et al*., 1996).

Nearly 10 years ago, a report claimed that TSST-1 was involved in the pathogenesis of Kawasaki disease, an acute febrile illness in infants and children with systemic clinical symptoms. As the clinical symptoms are quite similar in many respects with those of SAG-induced diseases, TSS, NTED, and *Y. ptbc*. infection, that report had an impact on our thinking. However, no further study supporting this contention was subsequently published. Likewise, we have found no evidence supporting this suggestion (Uchiyama, and Kato, 1999).

## Mode of *in vivo* T cell response to SAGs in humans and animals

SAG-induced expansions of human and mouse T cells are clearly demonstrated by *in vitro* experimental systems employing either $^3$H-thymidine uptake or PCR and immunological methods. However, we have long faced a discrepancy in the modes by which SAG-reactive T cells expand *in vivo* in mice versus humans. Analysis of the expansion of TSST-1-reactive Vβ2$^+$ T cells in TSS patients using the PCR method (Choi *et al*., 1990) suggested that Vβ2$^+$ T cells exhibited prolonged expansion, i.e., for more than 40 days, after onset of the illness. By comparison, in mice injected with SEA or SEB, which exhibit a level of T cell stimulating activity in human T cells *in vitro* similar to that of TSST-1, T cells reactive with these antigens exhibited only a transient expansion on day 2 or 3 after the injection, when examined using an immunological method (Rellahan *et al*., 1990; Kawabe and Ochi, 1991). The findings obtained in mouse experiments had been widely considered to reflect the *in vivo* response of T cells activated by SAGs. Over- or underestimation of Vβ2$^+$ T cells possibly associated with the semiquantitative PCR could not, however, explain this discrepancy. Recently, we have used

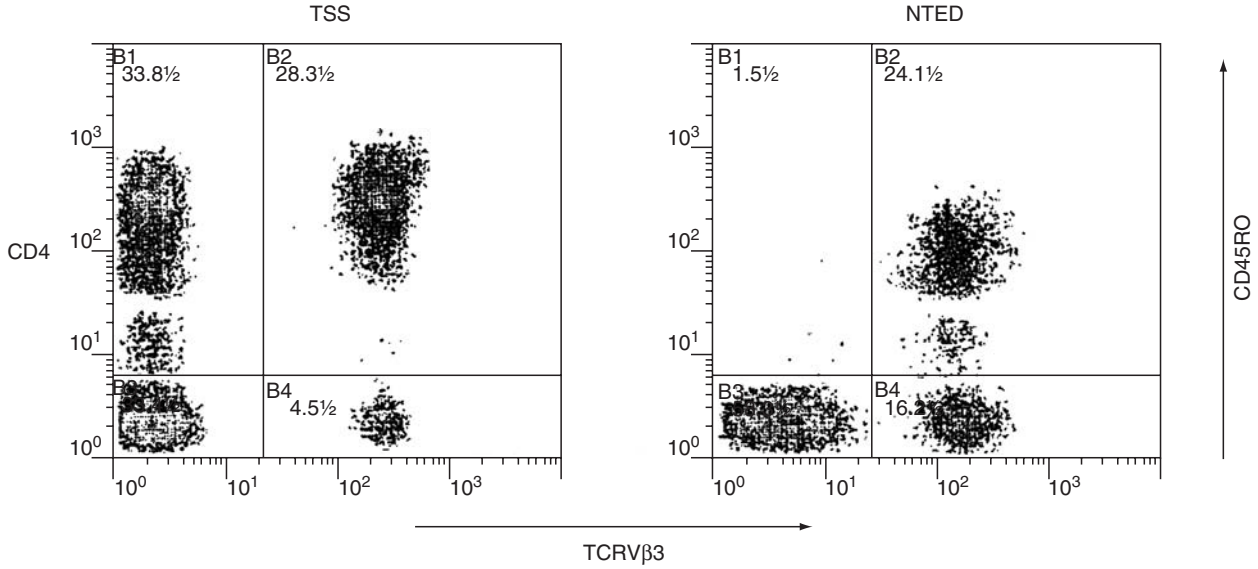



**FIGURE 50.2** Expansion of Vβ2$^+$ T cells in an adult patient with TSS and a neonate with NTED. Adult and neonatal patients manifesting acute and systemic clinical symptoms caused by MRSA were examined for the percentages of Vβ2$^+$CD4$^+$ T cells in PBM. Vβ2$^+$CD4$^+$ T cells comprised less than 10% of PBMs in healthy neonatal and adult controls. The percentages of CD45RO$^+$ cells among Vβ2$^+$ T cells are below 50% in healthy adults and below 5% in healthy neonates.

an immunologic method to observe the response patterns of Vβ2$^+$ T cells in PBM from two adult patients with TSS who contracted the illness as puerperal infection (Figure 50.3) (Matsuda *et al*., 2003). Both CD4$^+$Vβ2$^+$ T cells and CD8$^+$Vβ2$^+$ T cells in PBM expanded to 5 to 7 times the control level in the acute phase of the illness, and subsequently decreased gradually toward the control level over a period of 40 to 60 days or more, confirming the findings obtained by PCR.

It is reasonable to assume that adult patients with TSS have been exposed to TSST-1 continuously for a long period, while SAG-injected mice have been exposed to SAG for only a short period. The mechanism by which T cells are exposed to SAGs *in vivo* might account for the discrepancy. To address this question, C57BL/6 mice were injected with 10 μg of SEA or implanted with an osmotic pump containing 10 μg SEA, to be delivered continuously for approximately seven days. These mice were monitored for the percentages of SEA-reactive Vβ3$^+$ and Vβ11$^+$ T cells in splenic CD4/CD8 subsets at various time points after the treatments (Figure 50.4) (Chen *et al*., 2002). In mice injected with SEA, all four SEA-reactive T cell fractions uniformly exhibited a transient expansion, though at low levels, two days after injection. By comparison, in mice implanted with the SEA pump, massive Vβ3$^+$CD4$^+$ T cell expansion had occured by day 6 after implantation, and was maintained at comparably high levels for more than 10 days with only a slight subsequent reduction. Vβ11$^+$CD4$^+$ T cells had expanded moderately by day 6 after implantation and then decreased gradually to the control level, indicating that Vβ3+ T cells are highly responsve to SEA, while Vβ11$^+$CD4$^+$ T cells are moderately responsive. The SEA-reactive CD8$^+$ T cells exhibited only a transient expansion. In the aforementioned study of two TSS patients, the Vβ2$^+$CD8$^+$ fraction behaved in a manner similar to the CD4$^+$ fraction (Figure 50.3). However, in several other TSS cases Vβ2$^+$CD8$^+$ T cells responded weakly as compared with Vβ2$^+$CD4$^+$ T cells (Matsuda *et al*., manuscript in preparation). Findings were similar in patients with *Y. ptbc*. infection and mice implanted with an osmotic pump containing YPM. In the late acute phase patients, T cell expansion was observed only for Vβ3$^+$ T cells among three YPM-reactive (Vβ3$^+$, Vβ9$^+$, and Vβ13$^+$) human T cell populations (Abe *et al*., 1997). In the mouse experiments, Vβ7$^+$CD4$^+$ T cells corresponding to human Vβ3$^+$CD4$^+$ T cells exhibited prolonged expansion, while other YPM reactive CD4$^+$ T cell populations showed transient expansion (Chen *et al*., 2002). Taken together, these results indicate that the response pattern of SAG-reactive T cells *in vivo* differs markedly depending on which TCR Vβ elements the T cells express and the subsets of CD4/CD8. It appears likely that the Vβ2$^+$ T cells of the two TSST-1-reactive human cell populations respond strongly to TSST-1 *in vivo*.

## INFECTIOUS DISEASES CAUSED BY STAPHYLOCOCCAL SAGS

TSS and NTED have been established as being caused by staphylococcal SAGs. In this section, we will describe clinical features and early definitive diagnosis of these two diseases.

### TSS

#### *General description of TSS*

In 1978, Todd *et al*. originally reported a new disease entity characterized by multiple organ failure in seven children aged 8 to 17 manifesting high fever, profound hypotension, profuse diarrhea, skin rash, mental confusion, and renal failure (Todd *et al*., 1978).

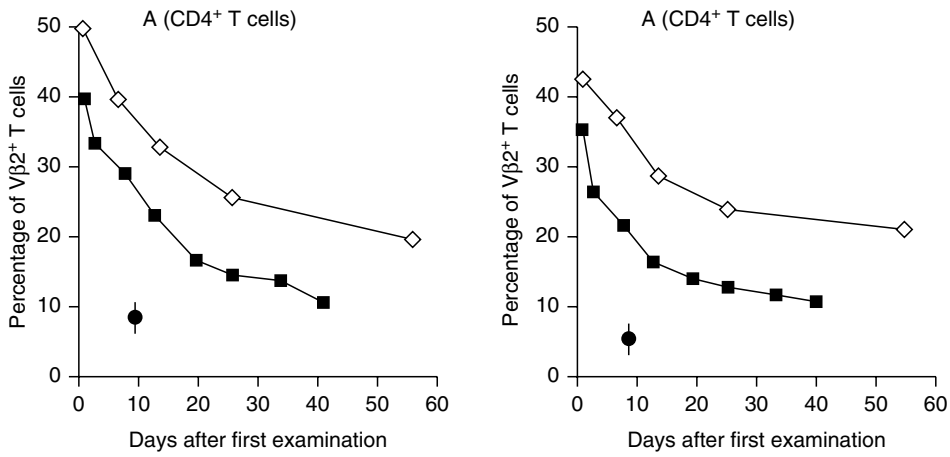



**FIGURE 50.3** Expansion of TCR Vb2$^+$ T cells in two women with TSS. The percentages of Vβ2 $^+$CD4$^+$ T cells (left panel) and Vβ2 $^+$CD8$^+$ T cells (right panel) in PBMs were determined since day 6 after disease onset in case 1 (closed squares), and day 5 after disease onset in case 2 (open squares). The two patients refer to the same cases as in the text and Table 50.2. ● represents mean ± standard deviation in healthy adults (four men and three women). From Matsuda *et al*., 2003.

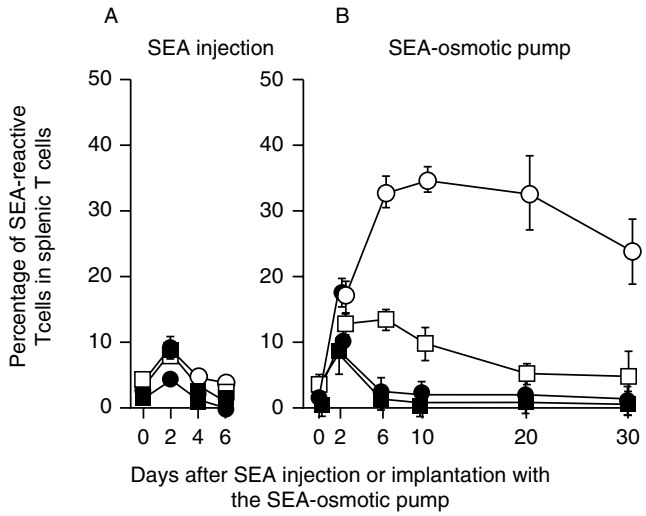



**FIGURE 50.4** TCR Vβ and CD4/CD8 subset-dependent expansion of SEA-reactive T cell fractions in mice implanted with SEA-osmotic pumps. C57BL/6 mice were injected with 10 μg of SEA (three to four per group) (A), or implanted with an osmotic pump containing 10 μg of SEA (six to eight per group) (B), and then monitored individually for the percentages of SEA-reactive T cells among splenic T cells (○). Vβ3 $^{+}$CD4$^{+}$ T cells; (●), Vβ3 $^{+}$CD8$^{+}$ T cells; (❒), Vβ11 $^{+}$CD4$^{+}$ T cells; (■), Vβ11$^{+}$CD8T cells. From chen *et al.*, 2002.

The disease was named toxic shock syndrome, because some of the patients were positive for *S. aureus* at foci of infection while blood, cerebrospinal fluid, or urine was negative, suggesting an association of the illness with a toxin from infectious organisms rather than direct invasion by these organisms. In the early 1980s, this multisystem disease was frequently observed in women with onset mainly during menstruation. The case fatality ratio was initially high, at 5–6%. Retrospective analysis has shown that around 1700 cases, mainly female patients whose onsets were during menstruation, were reported to the Centers for Disease Control and Prevention from 1970 to 1982. It seems likely that this major outbreak of TSS was related to a new type of tampon which had been contaminated with *S. aureus* (Todd, 1988). As to toxins causing TSS, TSST-1 was discovered in 1981, as described previously. Many papers have suggested that SEA, SEB, SEH, SEI, and SEJ in addition to TSST-1 are involved in the pathogenic mechanism of TSS, because *S. aureus* strains that produced the above toxins were isolated, without TSST-1, from some patients with TSS. These toxins were thus considered to have caused TSS. Surveillance in the United States showed that the incidence of TSS subsequently declined until 1996 (Hajjeh *et al.*, 1999). In recent years, however, the calculated incidence of TSS has reportedly been increasing from one case of TSS per month in 2000 to four cases per month in 2003 in an area with a population of approximately 3,000,000 (Schlievert *et al.*, 2004). In Japan no data are available as to whether the incidence of TSS is increasing. It would appear to be necessary to determine the TSS incidence rate in Japan, because we occasionally see TSS patients who manifest the illness as a puerperal infection.

We have observed several TSS patients who contracted the illness during the course of a puerperal infection. Some of these women manifested clinical symptoms that did not meet the clinical criteria for TSS, but the diagnosis of TSS was confirmed by determining the percentage of Vβ2$^{+}$ T cells in PBM.

### *Early definitive diagnosis of TSS*

In Japan, clinicians have described several puerperal infection cases caused by *S. aureus*, which suggested TSS, but could not be diagnosed as such according to either the strict clinical criteria for TSS (Shands *et al.*, 1989) or the revised criteria (Tofte and Williams, 1981). Diagnosis of TSS according to clinical features, irrespective of the strict or revised criteria, requires a minimum of several days' observation to obtain a reliable diagnosis. As TSS is often life-threatening, an early definitive diagnosis is crucial. We speculate that determining the percentage of Vβ2$^{+}$ T cells would provide a valuable clue to making a reliable diagnosis of TSS, because this method is based on the key pathogenic mechanism of TSS. Under this assumption, we have been monitoring the percentage of Vβ2$^{+}$ T cells in the PBMs of our patients using an immunological method. The patients investigated are women who manifest acute and systemic clinical symptoms as puerperal infections caused by *S. aureus*. Results obtained are sent to the wards within five hours after obtaining PBM specimens. Two TSS cases are presented in detail herein (Matsuda *et al.*, 2003). Clinical symptoms of case 1 were too severe and complex to permit diagnosis according to the clinical criteria without evaluation of Vβ2$^{+}$ T cells.

**Case 1:** A 29-year-old Japanese woman underwent cesarean section at a private clinic after premature membrane rupture. On postpartum day 3, shock with hypotension developed (day of disease onset), and she was transferred to our University Hospital. On admission, her body temperature was 39°C, systolic blood pressure 80 mmHg, respiratory rate 44 breaths /min., and heart rate 140 beats/min. She showed neither skin rash nor desquamation. MRSA, later confirmed to produce TSST-1, was detected in vaginal discharge. The clinical symptoms and laboratory data suggested a diagnosis of septic shock with disseminated intravascular coagulation (DIC). The clinical criteria for definite TSS and the revised form, as well as the

compatibility of her clinical symptoms and laboratory data with TSS are presented in Table 50.2. The clinical symptoms and laboratory data did not meet either set of criteria during her entire clinical course. On day 3 after admission (day 6 after disease onset), examination of Vβ2$^+$ T cells in PBM showed a marked expansion of Vβ2$^+$ T cells (Figure 50.3), indicating the illness to be TSS.

**Case 2:** A 33-year-old Japanese woman had a fever one week after an uncomplicated spontaneous vaginal delivery at a private clinic (day of disease onset). She was transferred to our University Hospital. On admission, her body temperature was 38.8°C, blood pressure 80/52. A diffuse erythematous rash starting on the chest had spread to the face and extremities, and resolved within eight days after admission. One day after admission, MRSA was isolated from vaginal discharge and breast milk. The clinical symptoms and laboratory data suggested a diagnosis of TSS. Examination of Vβ2$^+$ T cells on day 5 after admission (day 5 after disease onset) showed marked expansion of Vβ2$^+$ T cells, confirming the illness to be TSS.

Puerperal infection is a major cause of maternal death. Postpartum TSS has received attention as a potential cause of puerperal infection (Davis *et al*., 1998). Although we do not consider the incidence of TSS to be high in Japan, we are concerned that this disease is not rare in small private clinics. The immunological method described herein appears to be suitable for making an early definitive diagnosis of TSS.

**TABLE 50.2** Two types of clinical criteria for TSS and the compatibility to the criteria of the clinical symptoms and test results of two TSS cases

| Date | Case 1 | Case 2 |
|---|---|---|
| **Expansion of Vβ2$^+$ T cells** | + | + |
| **Critieria for definitive TSS** | – | + |
| (all criteria must be present) | | |
| ≥38.9°C | + | + |
| Rash with desquamation | – | + |
| Hypotension <90 mmHg | + | + |
| Clinical or laboratory abnormalities (≥3 organs) | | |
| Gastrointestinal | – | – |
| Hepatic | + | + |
| Muscular | – | – |
| Mucous membrane | – | + |
| Renal | + | + |
| Cardiovascular | – | – |
| Central nervous system | – | – |
| **Criteria for probable TSS** | – | + |
| (≥3 criteria and desquamation or ≥5 criteria without desquamation) | | |
| ≥38.9°C | + | + |
| Rash | – | + |
| Hypotension | + | + |
| Myalgia | – | – |
| Vomiting and/or diarrhea | – | – |
| Mucous membrane inflammation | – | + |
| Clinical or laboratory abnormalities (≥2 organs) | | |
| Gastrointestinal | – | – |
| Hepatic | + | + |
| Muscular | – | – |
| Mucous membrane | – | + |
| Renal | + | + |
| Cardiovascular | – | – |
| Central nervous system | – | – |

From Matsuda *et al*., 2003.

## Neonatal TSS-like exanthematous disease

### *General description of NTED*

In the mid-1990s, Takahashi *et al*. described a number of neonates who developed systemic exanthema, fever, low-positive serum C-reactive protein values, and thrombocytopenia within the first week of life, and described it as a new disease entity (Takahashi *et al*., 1995b). Many cases with this illness have been observed in hospitals in Japan since that report. Clinical symptoms and laboratory data of 20 patients are presented in Table 50.3 (Takahashi *et al*., 1998). A fever occurred on the second or third day of life and lasted for one day in most term infants, but was not seen in most of the preterm infants. Typical exanthema featuring a macular erythema spreading from the trunk to the face and extremities including the palms and soles lasted two to three days in term infants and two to six days in preterm infants. There was no desquamation, even during recovery. Platelet counts reach their lowest points on day 2–3 after the onset of exanthema in both preterm and term infants. CRP values were above the upper limit of normal in most patients. Microbiological tests showed all nasopharyngeal or umbilical swabs taken to be positive for TSST-1-producing MRSA. Blood cultures were all negative for MRSA, except in patient 20. Most term infants recover spontaneously without active treatment within a week of disease onset. Complications including apneic attacks and gastrointestinal signs were seen in many of the preterm infants, but were rare in the full-term neonates. Two of the preterm infants died as a result of tracheo-oesophageal fistula and necrotizing enterocolitis, respectively.

### *The pathogenic mechanism of NTED*

The findings of microbiological tests strongly suggested the pathogenic mechanism of this neonatal disease to be closely related to TSS, although clinical symptoms of most patients did not meet the clinical criteria for TSS. In a collaborative study, we theorized

that examination of the percentage of Vβ2$^+$ T cells in PBM T cells from patients might provide a clue to the pathogenesis of the neonatal disease.

PBM T cells from four of the 20 patients described in Table 50.3 during the acute and recovery phases and PBM T cells from four MRSA-free neonates on postnatal day 5 (control) were stained with Abs to Vβ2, Vβ3, Vβ9, Vβ12, and CD45, and examined for the percentages of T cell fractions expressing the respective Vβ elements using flow cytometry (Table 50.4) (Takahashi *et al*., 1998). It was found that the percentage of Vβ2$^+$ T cells was significantly higher in the four acute phase patients, and these percentages decreased to the control level during the recovery phase. The percentages of Vβ3$^+$, Vβ9$^+$, and Vβ12$^+$ T cells, which react with SEA, SEB, or SEC, were similar in the patients and controls, irrespective of the illness phase. Many of the Vβ2$^+$ T cells in the four patients expressed CD45RO in both acute and recovery phases. The results indicated that the illness was TSS in neonates with umbilical or nasopharyngeal MRSA colonization. As this neonatal disease could not be diagnosed as TSS, despite its pathogenesis being fundamentally the same as that of TSS, we proposed designating this disease neonatal TSS-like exanthematous disease (NTED) (Takahashi *et al*. 1998).

While monitoring the percentage of Vβ2$^+$ T cells in NTED patients after disease onset, we initially found that the mechanism of Vβ2$^+$ T cell expansion differed strikingly between adult patients with TSS and neonates with NTED. The percentages of Vβ2$^+$CD4$^+$ and Vβ2$^+$CD8+ T cells in NTED patients were high in the acute phase, but decreased rapidly approaching the con-

**TABLE 50.4** Expansion of Vβ2-positive T cells in NTED patients

| Patient | Age (days) | % of T cell expression Vβ2 | Vβ3 | Vβ9 | Vβ12 | % of Vβ2$^+$ T cells expressing CD45RO |
|---|---|---|---|---|---|---|
| Acute phase | | | | | | |
| 10 | 5 | 24.5 | 6.6 | 1.8 | 0.4 | 21.4 |
| 11 | 4 | 27.2 | 4.0 | 4.4 | 0.5 | 35.3 |
| 12 | 5 | 27.3 | 5.0 | 2.3 | 2.8 | 22.0 |
| 20 | 4 | 20.8 | 5.8 | 5.9 | 1.4 | 21.0 |
| 20 | 6 | 25.9 | 6.1 | 6.1 | 3.0 | 70.8 |
| Recovery phase | | | | | | |
| 10 | 19 | 9.1 | ND | ND | ND | 28.5 |
| 11 | 19 | 10.2 | 6.0 | 5.5 | 2.5 | 42.8 |
| Control | | | | | | |
| 1 | 5 | 8.9 | 5.7 | 3.9 | 1.8 | 0.4 |
| 2 | 5 | 12 | 8.2 | 1.9 | 2.3 | 0.2 |
| 3 | 5 | 10.8 | 8.3 | 3.7 | 0.8 | 0.1 |
| 4 | 5 | 8.1 | 6.8 | 2.9 | 0.8 | 0.2 |

PBM of patients 10, 11, 12, 20 were stained with antibodies to Vβ2, Vβ3, Vβ9, Vβ12, and CD45RO in several combinations and examind for the percentages of T cells using a flowcytometer. From Takahashi, *et al*., 1998.

**TABLE 50.3** Clinical profiles of neonatal patients with exanthema

| Patients | GA (wk) | BW (kg) | Exanthema postnatal days | Fever | Maximum CRP($\mu$g/l) | Minimum platelet (x10$^3$ $\mu$l) |
|---|---|---|---|---|---|---|
| Term infants | | | | | | |
| 1 | 39 | 3.5 | 4–6 | 3 | 24 | 99 |
| 2 | 41 | 3.2 | 3–5 | 3 | 59 | 54 |
| 3 | 39 | 2.8 | 2–5 | 2 | 13 | 99 |
| 4 | 38 | 2.9 | 3–5 | 2 | 16 | 107 |
| 5 | 37 | 2.6 | 4–6 | - | 26 | 56 |
| 6 | 41 | 4.2 | 3–4 | 2 | 40 | 115 |
| 7 | 40 | 3.2 | 4–6 | 3 | 30 | 31 |
| 8 | 40 | 3.1 | 3–5 | 2 | 64 | 52 |
| 9 | 41 | 2.8 | 2–5 | 3 | 66 | 78 |
| 10 | 39 | 3.0 | 4–6 | 3 | 8 | 60 |
| 11 | 39 | 2.8 | 3–5 | 2 | 64 | 94 |
| 12 | 40 | 2.9 | 3–5 | 3 | 26 | 16 |
| Preterm infants | | | | | | |
| 13 | 33 | 1.6 | 2–7 | - | 24 | 52 |
| 14 | 34 | 2.2 | 3–6 | - | 14 | 26 |
| 15 | 34 | 1.6 | 2–4 | 2 | 37 | 28 |
| 16 | 31 | 1.5 | 3–8 | - | 4 | 17 |
| 17 | 36 | 2.7 | 4–6 | - | 4 | 73 |
| 18 | 31 | 1.5 | 2–8 | - | 12 | 24 |
| 19 | 27 | 1.1 | 4–10 | - | 24 | 7 |
| 20 | 32 | 1.8 | 3–6 | - | 15 | 31 |
| Normal values | | | | | <10 | 359±119 |

From Takahashi *et al*., 1998.

trol level within 10 days and were within 10% of the control level by one or two months after disease onset (Figure 50.5) (Takahashi *et al*., 2000). By comparison, Vβ2$^+$ T cells in adult patients with TSS exhibited a prolonged expansion over 40–50 days after the disease onset (Figure 50.3). Reasons for the different response patterns seen in the two diseases will be discussed later. Next, we noticed that the percentage of Vβ2$^+$ T cells was occasionally quite low, dropping below the control level early in the illness, then increasing rapidly to 10 times the control level at the first examinations two or three days later (Kato *et al*., 2002). Currently, we consider T cells activated with TSST-1 to have disappeared from peripheral blood in the early acute phase due to being mobilized into lymph nodes or the spleen. As preterm infants with NTED manifest severe clinical symptoms and signs, the examination of Vβ2$^+$ T cells would be useful in making an early definitive diagnosis of NTED.

We have questioned whether T cells of asymptomatic MRSA carriers are affected by TSST-1. Examination of PBM T cells from 10 asymptomatic neonatal MRSA carriers on postnatal day 5 showed the percentages of Vβ2$^+$CD4$^+$ and Vβ2$^+$CD8$^+$T cells to be within normal range, but expression of CD45RO in Vβ2$^+$CD4$^+$ T cells increased in three of the 10 neonatal carriers (Takahashi *et al*., 2000), suggesting that Vβ2$^+$ T cells were only activated marginally by TSST-1 in some asymptomatic MRSA carriers.

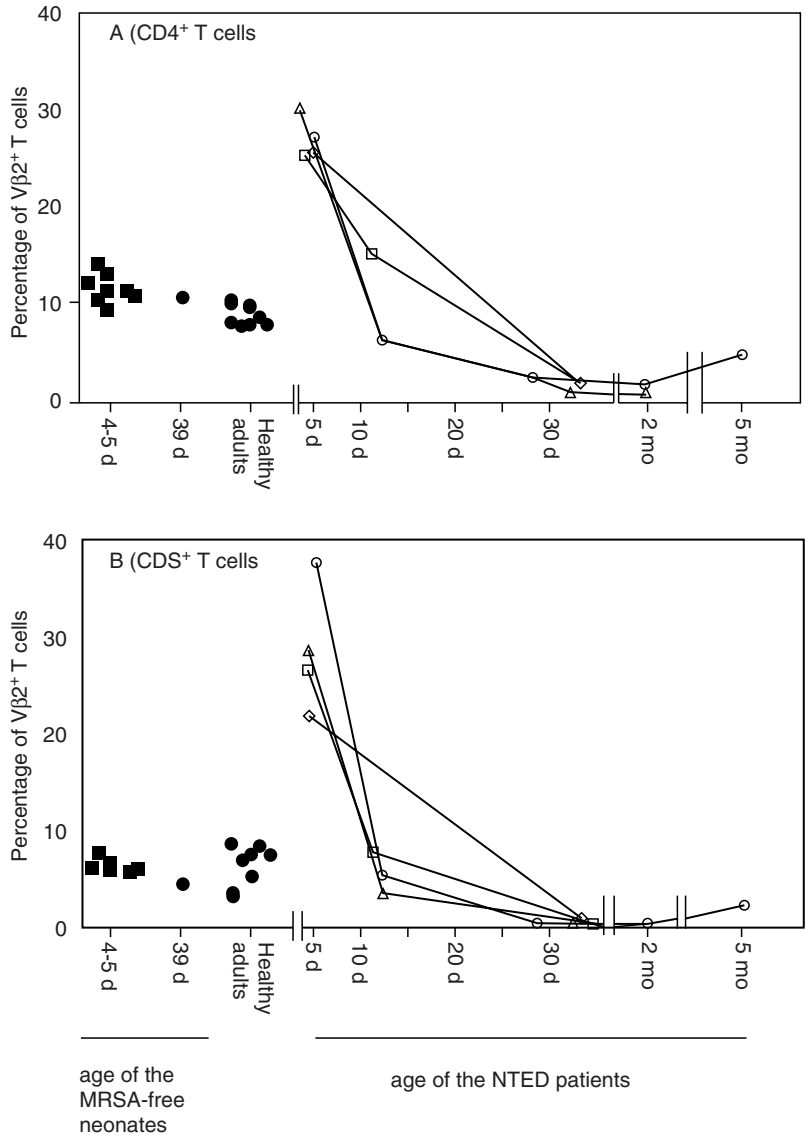



**FIGURE 50.5** Fate of TCR Vβ2$^+$ T cells in NTED patients. Four NTED patients (○, □, △,◇), nine MRSA-free neonates on postnatal days 5 (■) or 39 (◆), and seven healthy adults (●) were examined for the percentages of Vβ2 $^+$CD4$^+$ T cells (A) andVβ 2$^+$CD8$^+$ T cells (B) in PBMs. From Takahashi *et al*., 2000.

### *Protective role of maternal anti-TSST-1 Abs against development of NTED*

Anti-TSST-1 Abs are known to play a protective role against the development of TSS in adults. As neonates are generally protected from developing various diseases by specific Abs transferred transplacentally or by breast milk from their mothers, it is possible to speculate that a similar mechanism operates in neonates colonized with MRSA. Serum anti-TSST-1 IgG Abs were below detectable limits in the acute phase of the disease in all four NTED patients examined, but later rose markedly in three of these infants (Takahashi *et al*., 2000). In three of the 10 asymptomatic MRSA carriers with moderately activated $V\beta2^+$ T cells, the Abs were undetectable on postnatal day 5, and increased thereafter, while high on postnatal day 5 in four of the other seven asymptomatic carriers without activated $V\beta2^+$ T cells (Takahashi *et al*., 2000). These results indicate anti-TSST-1 IgG Abs transferred transplacentally to play a role in protecting neonates from NTED development.

## CORRELATION BETWEEN THE LEVEL OF T CELL ACTIVATION AND CLINICAL SYMPTOM SEVERITY IN TSS AND NTED

The view that TSST-1-induced T cell activation is primarily involved in generation of pathologic changes in TSS and NTED would appear to explain why the illness is severe in TSS and mild in NTED (Table 50.5). From the findings that the expanded state of $V\beta2^+$ T cells persists for a much longer period in adult patients with TSS than in neonates with NTED (Figs 50.3 and 50.5), it is possible that the magnitude of the T cell activation response to TSST-1 is much higher in the former than in the latter. The illness is quite severe in adult patients with TSS as compared with neonates with NTED. Cytokines, which have been strongly implicated in pathogenic changes, would be produced in much higher amounts in adult patients with TSS than in neonates with NTED. The clinical course of a term infant with NTED who manifested exceptionally severe clinical symptoms including DIC supports the above view. The expansion of $V\beta2^+$ T cells among PBMs from this patient was relatively prolonged (Miki *et al*., manuscript in preparation).

There are two possible explanations as to why the magnitude of the TSST-1-induced T cell response *in vivo* differs between adult and neonatal patients. First, the degree of T cell maturation is lower in neonates than in adults, resulting in the lower T cell response in NTED patients than in adult TSS patients.

**TABLE 50.5** T cell response to TSST-1 and clinical symptoms are quite different between neonates and adults

| | Neonates | Adults |
|---|---|---|
| Diseases induced by TSST-1 | NTED | TSS |
| Clinical symptoms | Not severe in most cases of term infants | Severe, often life-threatening |
| Fate of TSST-1-reactive T cells in patients | Transient expansion | Prolonged expansion |
| Maturition of T cells in the peripheral lymphoid organs | Immature | Maturated |

Alternatively, the relative amount of TSST-1 to which patients are exposed is much lower in the former than in the latter. According to Burnet's clonal selection theory, self-reactive lymphocytes in the neonatal period are eliminated upon encountering their corresponding self antigens because of their intrinsic immaturity (Burnet, 1959). Recent analyses have substantiated the above notion regarding human T cells (Takahashi *et al*., 1995; Imanishi *et al*., 1998; Kobayashi *et al*., 2003). For example, we have observed that cord blood $CD4^+$ T cells and human $CD1a^-CD4^+$ thymic T cells in the final stage of maturation in the thymus, which are believed to be the major source of cord blood T cells, were highly susceptible to anergy induction with TSST-1 *in vitro*, while $CD4^+$ T cells in adult PBM were highly resistant (Takahashi *et al*, 1995a; Imanishi *et al*., 1998). We have proposed that a defect in the interaction between the Src family kinase Lck and phosphatase CD45 after stimulation with antigens including SAGs is mainly responsible for the immaturity of neonatal T cells (Fujimaki *et al*., 2001). In most NTED patients, causative bacteria have been detected on the body surface but not in blood, while bacteria have often been detected in blood and damaged tissues in adult TSS patients, supporting the second possible explanation. These two factors may have worked synergistically to exacerbate the illness in adult patients while ameliorating neonates.

## REFERENCES

Abe, J., Takeda, T., Watanabe, Y., Nakao, H., Kobayashi, N., Leung, D.Y. and Kohsaka, T. (1993). Evidence for superantigen production by *Yersinia pseudotuberculosis*. *J. Immunol*. **151,** 4183–4188.

Abe, J., Onimaru, M., Matsumoto, S., Noma, S., Baba, K., Ito, Y., Kohsaka, T. and Takeda, T. (1997). Clinical role for a superantigen in *Yersinia pseudotuberculosis* infection. *J. Clin. Invest*. **99,** 1823–1830.

Alber, G., Hammer, D.K. and Fleischer, B. (1990). Relationship between enterotoxic and T lymphocyte-stimulating activity of staphylococcal enterotoxin B. *J. Immunol*. **144,** 4501–4506.

Alouf, J.E. and Muller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological, and clinical aspects. *Int. J. Med. Microbiol*. **292,** 429–440.

Arcus, V.L., Langley, R., Proft, T., Fraser, J.D. and Baker, E.N. (2002). The three-dimensional structure of a superantigen-like protein, SET3, from a pathogenicity island of the *Staphylococcus aureus* genome. *J. Biol. Chem*. **277,** 32274–32281.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K. and Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359,** 1819–1827.

Bayles, K.W. and Iandolo, J.J. (1989). Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. *J. Bacteriol*. **171,** 4799–4806.

Bergdoll, M.S. (1979). Staphylococcal intoxications. In: *Food-borne Infections and Intoxications* (ed. H. Riemann and F.L. Bryan). pp. 443–494. Academic Press, New York.

Bergdoll, M.S., Crass, B.A., Reiser, R.F., Robbins, R.N. and Davis, J.P. (1981). A new staphylococcal enterotoxin, enterotoxin F, associated with toxic shock syndrome *Staphylococcus aureus* isolates. *Lancet* **1,** 1017–1021.

Betley, M.J. and Mekalanos, J.J. (1988). Nucleotide sequence of the type A staphylococcal enterotoxin gene. *J. Bacteriol*. **170,** 34–41.

Blomster-Hautamaa, D.A., Kreiswirth, B.N., Kornblum, J.S., Novick, R.P. and Schlievert, P.M. (1986). The nucleotide and partial amino acid sequence of toxic shock syndrome toxin-1. *J. Biol. Chem*. **261,** 15783–15786.

Bohach, G.A. and Schlievert, P.M. (1987). Nucleotide sequence of the staphylococcal enterotoxin C1 gene and relatedness to other pyrogenic toxins. *Mol. Gen. Genet*. **209,** 15–20.

Bohach, G.A. and Schlievert, P.M. (1989). Conservation of the biologically active portions of staphylococcal enterotoxins C1 and C2. *Infect. Immun*. **57,** 2249–2252.

Bonventre, P.F., Weckbach, L., Staneck, J., Schlievert, P.M. and Thompson, M. (1983). Production of staphylococcal enterotoxin F and pyrogenic exotoxin C by *Staphylococcus aureus* isolates from toxic shock syndrome-associated sources. *Infect. Immun*. **40,** 1023–1029.

Burnet, F.M. (1959). *The Clonal Selection Theory of Acquired Immunity*. Cambridge University Press, Cambridge, United Kingdom.

Calvano, S.E., Quimby, F.W., Antonacci, A.C., Reiser, R.F., Bergdoll, M.S. and Dineen, P. (1984). Analysis of the mitogenic effects of toxic shock toxin on human peripheral blood mononuclear cells *in vitro*. *Clin. Immunol. Immunopathol*. **33,** 99–110.

Chen, L., Koyanagi, M., Fukada, K., Imanishi, K., Yagi, J., Kato, H., Miyoshi-Akiyama, T., Zhang, R., Miwa, K. and Uchiyama, T. (2002). Continuous exposure of mice to superantigenic toxins induces a high-level protracted expansion and an immunological memory in the toxin-reactive $CD4^+$ T cells. *J. Immunol*. **168,** 3817–3824.

Choi, Y., Lafferty, J.A., Clements, J.R., Todd, J.K., Gelfand, E.W., Kappler, J., Marrack, P. and Kotzin, B.L. (1990). Selective expansion of T cells expressing Vβ 2 in toxic shock syndrome. *J. Exp. Med*. **172,** 981–984.

Cohen, M.L., Graves, L.M., Hayes, P.S., Gibson, R.J., Rasheed, J.K. and Feeley, J.C. (1983). Toxic shock syndrome: modification and comparison of methods for detecting marker proteins in *Staphylococcus aureus*. *J. Clin. Microbiol*. **18,** 372–375.

Cole, B.C. and Atkin, C.L. (1991). The *Mycoplasma arthritidis* T-cell mitogen, MAM: a model superantigen. *Immunol. Today*. **12,** 271–276.

Couch, J.L., Soltis, M.T. and Betley, M.J. (1988). Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. *J. Bacteriol*. **170,** 2954–2960.

Crass, B.A. and Bergdoll, M.S. (1986). Involvement of coagulase-negative staphylococci in toxic shock syndrome. *J. Clin. Microbiol*. **23,** 43–45.

Davis, D., Gash-Kim, T.L. and Heffernan, E.J. (1998). Toxic shock syndrome: case report of a postpartum female and a literature review. *J. Emerg. Med*. **16,** 607–614.

Edwards, V.M., Deringer, J.R., Callantine, S.D., Deobald, C.F., Berger, P.H., Kapur, V., Stauffacher, C.V. and Bohach, G.A. (1997). Characterization of the canine type C enterotoxin produced by *Staphylococcus intermedius* pyoderma isolates. *Infect. Immun*. **65,** 2346–2352.

Fraser, J.D. (1989). High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. *Nature* **339,** 221–223.

Fujimaki, W., Iwashima, M., Yagi, J., Zhang, H., Yagi, H., Seo, K., Imai, Y., Imanishi, K. and Uchiyama, T. (2001). Functional uncoupling of T cell receptor engagement and Lck activation in anergic human thymic $CD4^+$ T cells. *J. Biol. Chem*. **276,** 17455–17460.

Gutterman, J.U., Blumenschein, G.R., Alexanian, R., Yap, H.Y., Buzdar, A.U., Cabanillas, F., Hortobagyi, G.N., Hersh, E.M., Rasmussen, S.L., Harmon, M., Kramer, M. and Pestka, S. (1980). Leukocyte interferon-induced tumor regression in human metastatic breast cancer, multiple myeloma, and malignant lymphoma. *Ann. Intern. Med*. **93,** 399–406.

Hajjeh, R.A., Reingold, A., Weil, A., Shutt, K., Schuchat, A. and Perkins, B.A. (1999). Toxic shock syndrome in the United States: surveillance update, 1979, 1996. *Emerg. Infect. Dis*. **5,** 807–810.

Harris, T.O. and Betley, M.J. (1995). Biological activities of staphylococcal enterotoxin type A mutants with N-terminal substitutions. *Infect. Immun*. **63,** 2133–2140.

Held, W., Acha-Orbea, H., MacDonald, H.R. and Waanders, G.A. (1994). Superantigens and retroviral infection: insights from mouse mammary tumor virus. *Immunol. Today*. **15,** 184–190.

Herrmann, T., Accolla, R.S. and MacDonald, H.R. (1989). Different staphylococcal enterotoxins bind preferentially to distinct major histocompatibility complex class II isotypes. *Eur. J. Immunol*. **19,** 2171–2174.

Hoffman, M., Tremaine, M., Mansfield, J. and Betley, M. (1996). Biochemical and mutational analysis of the histidine residues of staphylococcal enterotoxin A. *Infect. Immun*. **64**, 885–890.

Hovde, C.J., Hackett, S.P. and Bohach, G.A. (1990). Nucleotide sequence of the staphylococcal enterotoxin C3 gene: sequence comparison of all three type C staphylococcal enterotoxins. *Mol. Gen. Genet*. **220**, 329–333.

Hovde, C.J., Marr, J.C., Hoffmann, M.L., Hackett, S.P., Chi, Y.I., Crum, K.K., Stevens, D.L., Stauffacher, C.V. and Bohach, G.A. (1994). Investigation of the role of the disulphide bond in the activity and structure of staphylococcal enterotoxin C1. *Mol. Microbiol*. **13,** 897–909.

Imanishi, K., Igarashi, H. and Uchiyama, T. (1990). Activation of murine T cells by streptococcal pyrogenic exotoxin type A. Requirement for MHC class II molecules on accessory cells and identification of Vβ elements in T cell receptor of toxin-reactive T cells. *J. Immunol*. **145**, 3170–3176.

Imanishi, K., Seo, K., Kato, H., Miyoshi-Akiyama, T., Zhang, R.H., Takanashi, Y., Imai, Y. and Uchiyama, T. (1998). Post-thymic maturation of migrating human thymic single-positive T cells: thymic CD1a− CD4+ T cells are more susceptible to anergy induction by toxic shock syndrome toxin-1 than cord blood $CD4^+$ T cells. *J. Immunol*. **160,** 112–119.

Jarraud, S., Cozon, G., Vandenesch, F., Bes, M., Etienne, J. and Lina, G. (1999). Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J. Clin. Microbiol*. **37,** 2446–2449.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M. and Lina, G. (2001). *egc*,

a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus. J. Immunol*. **166,** 669–677; Correction (2001). *J. Immunol*. **166,** 4260.

Kappler, J., Kotzin, B., Herron, L., Gelfand, E.W., Bigler, R.D., Boylston, A., Carrel, S., Posnett, D.N., Choi, Y. and Marrack, P. (1989). Vβ-specific stimulation of human T cells by staphylococcal toxins. *Science* **244,** 811–813.

Kato, H., Takahashi, N., Arimura, Y., Imanishi, K., Nishida, H. and Uchiyama, T. (2002). The percentage of superantigen-reactive T cells in peripheral blood significantly decreases before massively increasing in patients with neonatal TSS-like exanthematous disease in the early acute phase. *J. Infect. Chemother*. **8**, 111–114.

Kawabe, Y. and Ochi, A. (1991). Programmed cell death and extrathymic reduction of $V\beta8^+$ $CD4^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature* **349,** 245–248.

Kobayashi, S., Ohnuma, K., Uchiyama, M., Iino, K., Iwata, S., Dang, N.H. and Morimoto, C. (2004). Association of CD26 with CD45RA outside lipid rafts attenuates cord blood T cell activation. *Blood* **103,** 1002–1010.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramats, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus. Lancet* **357,** 1225–1240.

Larkin, S.M., Williams, D.N., Osterholm, M.T., Tofte, R.W. and Posalaky, Z. (1982). Toxic shock syndrome: clinical, laboratory, and pathologic findings in nine fatal cases. *Ann. Intern. Med*. **96,** 858–864.

Letertre, C., Perelle, S., Dilasser, F. and Fach, P. (2003). Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus. J. Appl. Microbiol*. **95,** 38–43.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. and Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by *Staphylococcus. J. Infect. Dis*. **189,** 2334–2336.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N. and Novick, R.P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus. Mol. Microbiol*. **29,** 527–543.

Lotze, M.T., Matory, Y.L., Ettinghausen, S.E., Rayner, A.A., Sharrow, S.O., Seipp, C.A., Custer, M.C. and Rosenberg, S.A. (1985). *In vivo* administration of purified human interleukin 2. II. Half life, immunologic effects, and expansion of peripheral lymphoid cells *in vivo* with recombinant IL 2. *J. Immunol*. **135,** 2865–2875.

Marrack, P., Blackman, M., Kushnir, E. and Kappler, J. (1990). The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J. Exp. Med*. **171,** 455–464.

Matsuda, Y., Kato, H., Yamada, R., Okano, H., Ohta, H., Imanishi, K., Kikuchi, K., Totsuka, K. and Uchiyama, T. (2003). Early and definitive diagnosis of toxic shock syndrome by detection of marked expansion of T cell receptor Vβ2-positive T cells. *Emerg. Infect. Dis*. **9,** 387–389.

McCormick, J.K., Yarwood, J.M. and Schlievert, P.M. (2001). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55,** 77–104.

Miyoshi-Akiyama, T., Abe, A., Kato, H., Kawahara, K., Narimatsu, H. and Uchiyama, T. (1995). DNA sequencing of the gene encoding a bacterial superantigen, *Yersinia pseudotuberculosis*-derived mitogen (YPM), and characterization of the gene product, cloned YPM. *J. Immunol*. **154,** 5228–5234.

Mollick, J.A., Cook, R.G. and Rich, R.R. (1989). Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. *Science* **244,** 817–820.

Munson, S.H., Tremaine, M.T., Betley, M.J. and Welch, R.A. (1998). Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus. Infect. Immun*. **66,** 3337–3348.

Omoe, K., Hu, D.L., Takahashi-Omoe, H., Nakane, A. and Shinagawa, K. (2003). Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect. Immun*. **71,** 6088–6094.

Omoe, K., Imanishi, K., Hu, D.L., Kato, H., Takahashi-Omoe, H., Nakane, A., Uchiyama, T. and Shinagawa, K. (2004). Biological properties of staphylococcal enterotoxin-like toxin type R. *Infect. Immun*. **72,** 3664–3667.

Omoe, K., lmanishi, K., Mu, D.L., Kato, H., Fugane, Y., Abe, Y., Hamaoka, S., Watanabe, Y., Nakane, A., Uchiyama, T., and Shinagawa, K. *Infect Immun.* 2005. (In press).

Orwin, P.M., Leung, D.Y., Donahue, H.L., Novick, R.P. and Schlievert, P.M. (2001). Biochemical and biological properties of Staphylococcal enterotoxin K. *Infect. Immun*. **69,** 360–366.

Orwin, P.M., Leung, D.Y., Tripp, T.J., Bohach, G.A., Earhart, C.A., Ohlendorf, D.H. and Schlievert, P.M. (2002). Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* **41,** 14033–14040.

Orwin, P.M., Fitzgerald, J.R., Leung, D.Y., Gutierrez, J.A., Bohach, G.A. and Schlievert, P.M. (2003). Characterization of *Staphylococcus aureus* enterotoxin L. *Infect. Immun*. **71,** 2916–2919.

Paris, A.L., Herwaldt, L.A., Blum, D., Schmid, G.P., Shands, K.N. and Broome, C.V. (1982). Pathologic findings in twelve fatal cases of toxic shock syndrome. *Ann. Intern. Med*. **96,** 852–857.

Parsonnet, J., Gillis, Z.A., Richter, A.G. and Pier, G.B. (1987). A rabbit model of toxic shock syndrome that uses a constant, subcutaneous infusion of toxic shock syndrome toxin 1. *Infect. Immun*. **55,** 1070–1076.

Petersson, K., Pettersson, H., Skartved, N.J., Walse, B. and Forsberg, G. (2003). Staphylococcal enterotoxin H induces Vα-specific expansion of T cells. *J. Immunol*. **170,** 4148–4154.

Poindexter, N.J., and Schlievert, P.M. (1985). Toxic shock syndrome toxin 1-induced proliferation of lymphocytes: comparison of the mitogenic response of human, murine, and rabbit lymphocytes. *J. Infect. Dis*. **151,** 65–72.

Proft, T. and Fraser, J.D. (2003). Bacterial superantigens. *Clin. Exp. Immunol*. **133,** 299–306.

Ranelli, D.M., Jones, C.L., Johns, M.B., Mussey, G.J. and Khan, S.A. (1985). Molecular cloning of staphylococcal enterotoxin B gene in *Escherichia coli* and *Staphylococcus aureus. Proc. Natl. Acad. Sci. USA* **82,** 5850–5854.

Rasheed, J.K., Arko, R.J., Feeley, J.C., Chandler, F.W., Thornsberry, C., Gibson, R.J., Cohen, M.L., Jeffries, C.D. and Broome, C.V. (1985). Acquired ability of *Staphylococcus aureus* to produce toxic shock-associated protein and resulting illness in a rabbit model. *Infect. Immun*. **47,** 598–604.

Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., Fry, A.M. and Matis, L.A. (1990). *In vivo* induction of anergy in peripheral V beta 8+ T cells by staphylococcal enterotoxin B. *J. Exp. Med*. **172,** 1091–1100.

Ren, K., Bannan, J.D., Pancholi, V., Cheung, A.L., Robbins, J.C., Fischetti, V.A. and Zabriskie, J.B. (1994). Characterization and biological properties of a new staphylococcal exotoxin. *J. Exp. Med*. **180,** 1675–1683.

Schlievert, P.M., Shands, K.N., Dan, B.B., Schmid, G.P. and Nishimura, R.D. (1981). Identification and characterization of an

exotoxin from *Staphylococcus aureus* associated with toxic shock syndrome. *J. Infect. Dis*. **143,** 509–516.

Schlievert, P.M., Tripp, T.J. and Peterson, M.L. (2004). Reemergence of staphylococcal toxic shock syndrome in Minneapolis-St. Paul, Minnesota, during the 2000–2003 surveillance period. *J. Clin. Microbiol*. **42,** 2875–2876.

Scholl, P., Diez, A., Mourad, W., Parsonnet, J., Geha, R.S. and Chatila, T. (1989). Toxic shock syndrome toxin 1 binds to major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA* **86,** 4210–4214.

Shands, K.N., Schmid, G.P., Dan, B.B., Blum, D., Guidotti, R.J., Hargrett, N.T., Anderson, R.L., Hill, D.L., Broome, C.V., Band, J.D. and Fraser, D.W. (1980). Toxic shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases. *N. Engl. J. Med*. **303,** 1436–1442.

Su, Y.C. and Wong, A.C. (1995). Identification and purification of a new staphylococcal enterotoxin, H. *Appl. Environ. Microbiol*. **61,** 1438–1443.

Takahashi, N., Imanishi, K., Nishida, H. and Uchiyama, T. (1995a). Evidence for immunologic immaturity of cord blood T cells. Cord blood T cells are susceptible to tolerance induction to *in vitro* stimulation with a superantigen. *J. Immunol*. **155,** 5213–5219.

Takahashi, N., Nishida, H., Ino, M., Sakata, Y., Sakuma, I. and Takeda, Y. (1995b). A new exanthematous disease in newborn infants. *Acta Neonatal. Jpn*. **31,** 371–377.

Takahashi, N., Nishida, H., Kato, H., Imanishi, K., Sakata, Y. and Uchiyama, T. (1998). Exanthematous disease induced by toxic shock syndrome toxin 1 in the early neonatal period. *Lancet* **351,** 1614–1619.

Takahashi, N., Kato, H., Imanishi, K., Miwa, K., Yamanami, S., Nishida, H. and Uchiyama, T. (2000). Immunopathophysiological aspects of an emerging neonatal infectious disease induced by a bacterial superantigen. *J. Clin. Invest*. **106,** 1409–1415.

Todd, J., Fishaut, M., Kapral, F. and Welch, T. (1978). Toxic shock syndrome associated with phage group-I *Staphylococci. Lancet* **2,** 1116–1118.

Todd, J.K. (1988). Toxic shock syndrome. *Clin. Microbiol. Rev*. **1,** 432–446.

Tofte, R.W. and Williams, D.N. (1981). Toxic shock syndrome. Evidence of a broad clinical spectrum. *JAMA* **246,** 2163–2167.

Uchiyama, T., Kamagata, Y., Wakai, M., Yoshioka, M., Fujikawa, H. and Igarashi, H. (1986). Study of the biological activities of toxic shock syndrome toxin-1. I. Proliferative response and interleukin 2 production by T cells stimulated with the toxin. *Microbiol. Immunol*. **30,** 469–483.

Uchiyama, T., Kamagata, Y., Yan, X.J., Kohno, M., Yoshioka, M., Fujikawa, H., Igarashi, H., Okubo, M., Awano, F., Saito-Taki, T. and Nakano, M. (1987). Study of the biological activities of toxic shock syndrome toxin-1: II. Induction of the proliferative response and the interleukin 2 production by T cells from human peripheral blood mononuclear cells stimulated with the toxin. *Clin. Exp. Immunol*. **68,** 638–647.

Uchiyama, T., Imanishi, K., Saito, S., Araake, M., Yan, X.J., Fujikawa, H., Igarashi, H., Kato, H., Obata, F., Kashiwagi, N. and Inoko, H. (1989a). Activation of human T cells by toxic shock syndrome toxin-1: the toxin-binding structures expressed on human lymphoid cells acting as accessory cells are HLA class II molecules. *Eur. J. Immunol*. **19,** 1803–1809.

Uchiyama, T., Tadakuma, T., Imanishi, K., Araake, M., Saito, S., Yan, X.J., Fujikawa, H., Igarashi, H. and Yamaura, N. (1989b). Activation of murine T cells by toxic shock syndrome toxin-1. The toxin-binding structures expressed on murine accessory cells are MHC class II molecules. *J. Immunol*. **143,** 3175–3182.

Uchiyama, T., Miyoshi-Akiyama, T., Kato, H., Fujimaki, W., Imanishi, K. and Yan, X.J. (1993). Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* isolated from patients manifesting acute and systemic symptoms. *J. Immunol*. **151,** 4407–4413.

Uchiyama, T., Yan, X.J., Imanishi, K. and Yagi, J. (1994). Bacterial superantigens—mechanism of T cell activation by the superantigens and their role in the pathogenesis of infectious diseases. *Microbiol. Immunol*. **38,** 245–256.

White, J., Herman, A., Pullen, A.M., Kubo, R., Kappler, J.W. and Marrack, P. (1989). The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56,** 27–35.

Williams, R.J., Ward, J.M., Henderson, B., Poole, S., O'Hara, B.P., Wilson, M. and Nair, S.P. (2000). Identification of a novel gene cluster encoding staphylococcal exotoxin-like proteins: characterization of the prototypic gene and its protein product, SET1. *Infect. Immun*. **68,** 4407–4415.

Zhang, S., Iandolo, J.J. and Stewart, G.C. (1998). The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (sej). *FEMS Microbiol. Lett*. **168,** 227–233.

CHAPTER

# 51

# Streptococcal superantigenic toxins

*Thomas Proft and John D. Fraser*

## INTRODUCTION

*Streptococcus pyogenes* (or Lancefield group A streptococcus, GAS) is a Gram-positive commensal bacterium that colonizes human tissue. It is estimated that between 5–15% of healthy individuals harbor the bacterium, usually in the respiratory tract, without causing disease. However, when the host immune system is compromised or when the bacteria are able to penetrate the physical barrier of the skin (e.g., wound infection), *S. pyogenes* can cause a wide range of diseases. Mild GAS diseases include pharyngitis and tonsillitis, which occur primarily among children 5–15 years of age and in confined institutions, like military camps. If untreated, these diseases can develop into severe conditions such as acute rheumatic fever (ARF), rheumatic heart disease (RHD), and poststreptococcal acute glomerulonephritis (Cunningham, 2000; McDonald *et al*., 2004). Penetration of the skin barrier by GAS can cause bacteremia and severe invasive deep tissue infections, such as cellulitis, myositis, and necrotizing fasciitis (also known as the "flesh-eating disease") (Stevens, 1992, 2000; Seal, 2001; McCormick *et al*., 2001b).

During the late nineteenth century, GAS caused epidemic outbreaks of scarlet fever and ARF in Europe and in Northern America. After World War II, incidence rates for scarlet fever and ARF sharply declined and by the 1970s reached their lowest point. However, since the 1980s there has been an alarming increase in GAS-mediated invasive disease, not only in frequency, but also in severity.

The most severe form of invasive infection results in clinical symptoms similar to staphylococcal toxic shock syndrome and was therefore described as streptococcal toxic shock syndrome (STSS). This life-threatening disease (30–70% mortality) was first recognized in 1983 (Willoughby and Greenberg, 1983) and has been observed worldwide since 1987 (Stevens 1992, 1995, 2000; McCormick *et al*., 2001). Characteristic symptoms of STSS include hypotension, fever, rash, vomiting, diarrhea, multiple organ failure, and shock (Working Group on Severe Streptococcal Infections, 1993).

It is widely believed that the resurgence of invasive GAS infections is the result of an interplay between changes in bacterial virulence and host susceptibility (Schwartz *et al*., 1990). The acquisition of novel virulence factors might have resulted in the development of more aggressive GAS strains (Bisno *et al*., 2003). Virulence factors include several adhesion molecules and a large number of secreted proteins (over 30) comprising the streptococcal superantigens (this chapter), cytolysins, proteases, DNAses, and various hydrolytic enzymes.

## SUPERANTIGENS

The term "superantigen" (SAg) was coined in 1989 by the research group of Philippa Marrack and John Kappler and describes a group of proteins with extremely high potency to stimulate human, and to a certain degree, other mammalian CD4 and CD8 T cells (White *et al*, 1989).

Unlike conventional peptide antigens, SAgs bind to major histocompatibility class II (MHC II) molecules outside the peptide-binding groove and are presented as unprocessed molecules to T cells carrying a T cell receptor (TcR) with a particular Vβ region (Dellabona

*et al*., 1990; Herman *et al*., 1991; Seth *et al*., 1994). Since the number of different Vβ regions in the human T cell repertoires is restricted to less than 50, comprising about 24 major types of Vβ elements, and since most SAgs can bind more than one Vβ, up to 25% of an individual's T cell population can be activated, resulting in massive release of pro-inflammatory cytokines and T cell mediators (Herman *et al*., 1991; Miethke *et al*., 1992; Hackett and Stevens, 1993; Müller-Alouf *et al*., 1996). In contrast, conventional peptide presentation results in activation of only one in $10^5$–$10^6$ T cells (0.001–0.0001%). This makes SAgs the most powerful T cell mitogens ever discovered. Concentrations of less than 0.1 pg/ml of a bacterial superantigen are sufficient to stimulate the T lymphocytes in an uncontrolled manner, resulting in fever, shock, and death (Jupin *et al*., 1988; Fast *et al*., 1989; Herman *et al*., 1991; Miethke *et al*., 1992; Lavoie *et al*., 1999; Bohach *et al*., 1990; Marrack and Kappler, 1990; Fraser *et al*., 2000; McCormick *et al*., 2001b).

There are two major categories of SAgs: the viral SAgs and the bacterial SAgs. Viral SAgs comprise the minor lymphocyte stimulating (Mls) antigens from the mouse mammary tumor virus (MMTV) and the recently discovered SAgs encoded by human endogenous retroviruses.

The bacterial SAgs are small, secreted exotoxins (15–28 kDa) produced by *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus equi*, *Streptococcus disgalactiae*, *Yersinia pseudotuberculosis*, and *Mycoplasma arthritidis.*

## GROUP A STREPTOCOCCAL SUPERANTIGENS

### Discovery and nomenclature

The discovery of streptococcal superantigens can be divided into two major periods: the pre-genomic era and the post-genomic era. During the pre-genomic era, streptococcal SAgs were identified in a cell culture supernatant based on their mitogenic activity. The toxins were biochemically enriched to a purity that was sufficient for functional studies and for analysis by N-terminal sequencing. In the post-genomic era, novel streptococcal SAgs were identified by database mining of GAS genomes and recombinant forms of the toxins were used for functional studies. Eleven GAS superantigens are currently known, but the number will most likely increase when more GAS genomes are analyzed. The GAS superantigens share amino acid sequence identities between 48% (SPE C and SPE J) and 17% (SSA and SPE L/M) (Figure 51.1). Furthermore, the streptococcal SAgs are part of an even larger family together with the structurally related staphylococcal SAgs.

#### *The pre-genomic GAS superantigens*

In 1924, Dick and colleagues intradermically injected culture filtrates from hemolytic streptococci of scarlatinal origin into healthy volunteers and observed an erythematous and edematous skin reaction in susceptible individuals 24 hours after injection (Dick and Dick, 1924). The injection of larger doses induced scarlet fever–like symptoms (also called miniature scarlet fever) with fever, nausea, vomiting, and a transient scarlatiniform rash. They postulated that this reaction (also known as the "Dick reaction") was caused by an erythrogenic toxin, which they named "scarlet fever toxin." However, it was later suggested that the skin rash resulted from a hypersensitivity reaction to a number of different streptococcal products and not alone from erythogenic toxin (Kim and Watson, 1970; Schlievert *et al*., 1979).

A second toxin (toxin B) was identified in 1934 (Hooker and Follensby, 1934). Toxin B, which is immunologically different from erythrogenic toxin A, was later purified and biochemically characterized by Stock and Lynn (1961).

In 1960, Watson discovered yet another toxin that was immunologically different from toxins A and B. Toxin C was isolated from a culture filtrate of a strain (serotype M18) associated with scarlet fever (Watson, 1960). By the 1970s, several different biological activities had been described for the three toxins, in particular the ability to induce fever when injected into rabbits (pyrogenicity) and the enhancement of animal susceptibility to lethal endotoxic shock. Since pyrogenicity was believed to be the primary effect of the toxins, Kim and Watson designated the toxins as streptococcal pyrogenic exotoxins A, B, and C (SPE A,B,C) (Kim and Watson, 1970).

During the late 1980s, the three toxin genes were cloned in *E. coli* and recombinant forms were produced. The *speA* gene was cloned from bacteriophage T12, and DNA analysis revealed an open reading frame consisting of 756 base pairs encoding a 252 amino acid precurser protein (Weeks and Ferretti, 1986). The N-terminal 30 amino acids represent a signal peptide that is removed during secretion to produce the mature toxin. Recombinant SPE A was produced in *Bacillus subtilis* and shown to mimic the biological activity of native preparations (Kreiswirth *et al*., 1987). A novel biological activity for SPE A was demonstrated by Schlievert and Grey (1989), who found that SPE A is identical to a previously identified T cell mitogen, Blastogen A. One year later, it was demonstrated that SPE A activates murine T cells in a MHC II-dependent and TcRVβ-specific mode, finally establishing its function as a superantigen (Imanishi *et al*., 1990).

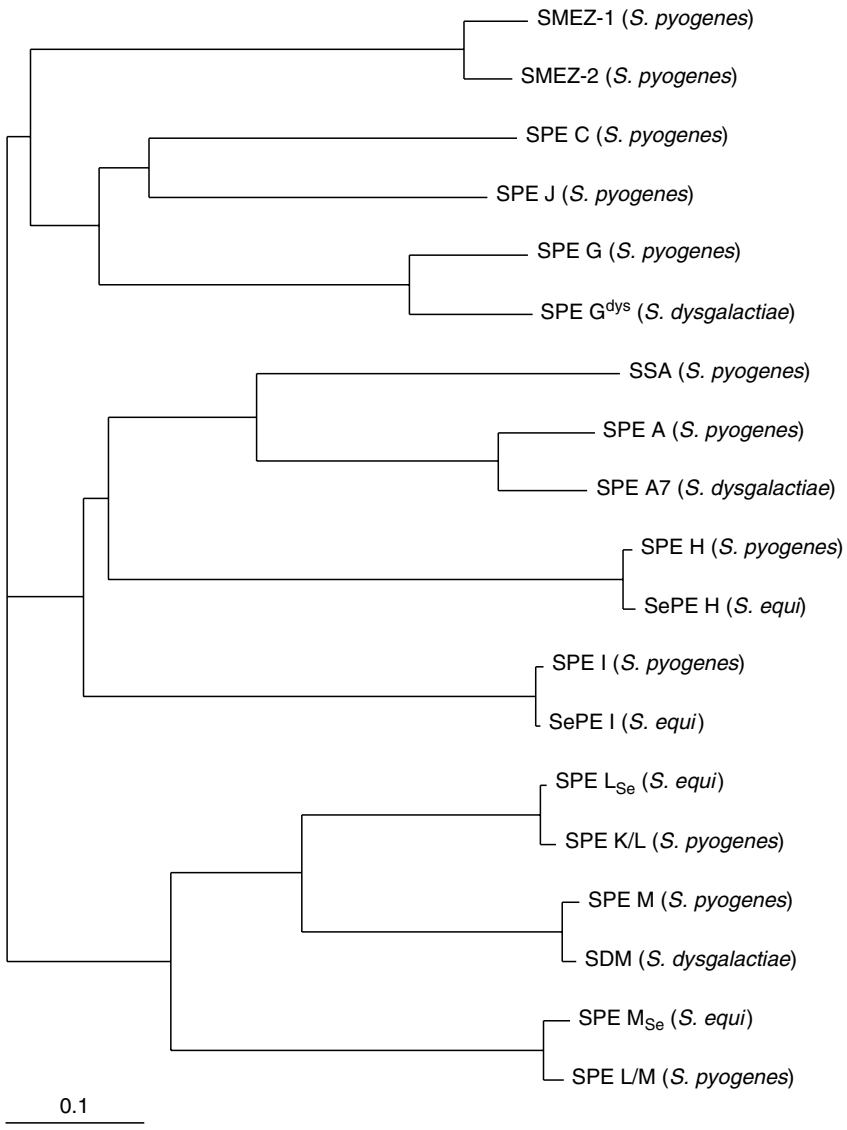



**FIGURE 51.1** Streptococcal superantigen family tree. The tree was created using Clustal W and TreeViewPPC version 1.5.3 and is based on primary amino acid sequence homology of the mature toxins.

The *speB* gene was cloned from a serotype M12 GAS strain (Bohach *et al*., 1988) and shown to be identical to streptococcal cysteine protease (SCP), confirming earlier results that showed immunological cross-reactivity between SPE B and SCP (Gerlach *et al*., 1983). Earlier reports that demonstrated SPE B induced T cell proliferation (Abe *et al*., 1991) were later disputed by others, who used SPE B preparations of very high purity and argued that the initial findings were due to contamination with an unknown superantigen (Braun *et al*., 1993; Gerlach *et al*., 1994). It is now widely accepted that SPE B is not a superantigen.

The *speC* gene was cloned from the chromosome of *S. pyogenes* strain T18P by Goshorn *et al*. (1988). The status of SPE C as superantigen was established in 1991, when Leonard *et al*. (1991) showed MHC II/TcRVβ-dependent T cell mitogenicity.

A novel superantigen was discovered in the cell culture supernatant of *S. pyogenes* strain Weller (serotype M3) and named streptococcal superantigen (SSA) (Mollick *et al*., 1993). SSA showed a higher degree of amino acid sequence similarity to the staphylococcal enterotoxins B, C1, and C3 (SEB, SEC1, and SEC3) than to SPE A or SPE C. SSA targets T cells bearing the Vβ1, 3, and 15 regions, in contrast to SPE A, which mainly targets Vβ12 and 14, or SPE C, which mainly targets Vβ2 (Table 51.1).

Yet another novel SAg was reported by Kamezawa *et al*. (1997) and named streptococcal mitogenic exotoxin Z (SMEZ). This toxin was purified from *S. pyogenes* serotype M1/T1 strain and showed a unique TcR binding specificity targeting mainly Vβ4 and 8 regions. An allelic variant of this toxin was later purified from an *S. pyogenes* serotype T12 strain and designated SPE-X/SMEZ3 (Gerlach *et al*., 2000).

**TABLE 51.1** Functional properties of streptococcal superantigens

| SAg | MW [kDa] | Organism | Crystal structure | Zinc binding | MHC II binding α/β chain | Human TcR Vβ specificity | $P_{50}$ (h) [pg/ml] | Reference |
|---|---|---|---|---|---|---|---|---|
| SPE A | 26.0 | *S. pyogenes* | + | + | +/− | 2.1, *12.2*, *14.1*, 15.1 | ? | Weeks and Ferretti, 1986; Abe *et al.*, 1991 |
| SPE C | 24.4 | *S. pyogenes* | + | + | −/+ | *2.1*, 3.2, 12.5, 15.1 | 0.1 | Goshorn *et al.*, 1988; Li *et al.*, 1998 |
| SPE G | 24.6 | *S. pyogenes* | − | + | −/+ | *2.1*, 4.1, 6.9, 9.1, 12.3 | 2 | Proft *et al.*, 1999 |
| SPE H | 23.6 | *S. pyogenes* | + | + | −/+ | 2.1, *7.3*, 9.1, 23.1 | 50 | Proft *et al.*, 1999 |
| SPE I | 26.0 | *S. pyogenes* | − | + | −/+ | 6.9, 9.1, *18.1*, 22 | 0.1 | Proft *et al.*, 2001; Ferretti *et al.*, 2001 |
| SPE J | 24.6 | *S. pyogenes* | + | + | −/+ | *2.1* | 0.1 | Proft *et al.*, 2001; McCormick *et al.*, 2001a |
| SPE K/L | 27.4 | *S. pyogenes* | − | + | −/+ | *1.1*, 5.1, 23.1 | 1 | Beres *et al.*, 2002; Ikebe *et al.*, 2002; Proft *et al.*, 2003b |
| SPE L/M | 26.2 | *S. pyogenes* | − | + | −/+ | *1.1*, 5.1, 23.1 | 10 | Smoot *et al.*, 2002a; Proft *et al.*, 2003b |
| SPE M | 25.3 | *S. pyogenes* | − | + | ? | *1.1*, 5.1, 23.1 | ? | Smoot *et al.*, 2002a |
| SSA | 26.9 | *S. pyogenes* | + | − | +/− | 1.1, 3, 15 | ? | Mollick *et al.*, 1993; Stevens K., *et al.*, 1996 |
| SMEZ-1 | 24.3 | *S. pyogenes* | − | + | −/+ | 2.1, *4.1*, 7.3, *8.1* | 0.08 | Kamezawa *et al.*, 1997; Proft *et al.*, 1999 |
| SMEZ-2 | 24.1 | *S. pyogenes* | + | + | −/+ | *4.1*, *8.1* | 0.02 | Proft *et al.*, 1999 |
| SePE H | 23.6 | *S. equi* | − | + | ? | ? | ? | Artuishin *et al.*, 2002 |
| SePE I | 25.7 | *S. equi* | − | + | ? | ? | ? | Artuishin *et al.*, 2002 |
| SPE $L_{Se}$ | 27.4 | *S. equi* | − | + | ? | ? | ? | Proft *et al.*, 2003b |
| SPE $M_{Se}$ | 26.2 | *S. equi* | − | + | ? | ? | ? | Proft *et al.*, 2003b |
| SPE A-7 | 25.9 | *S. dysgalactiae* | − | + | ? | ? | ? | Kalia and Bessen, 2003 |
| SPE $G^{dys}$ | 24.4 | *S. dysgalactiae* | − | + | ? | ? | ? | Sachse *et al.*, 2002 |
| SDM | 25.0 | *S. dysgalactiae* | − | + | ? | 1.1, 23 | ? | Miyoshi *et al.*, 2003 |

The major TcRVβ targets are italicized and underlined.

### *The post-genomic GAS superantigens*

The completion of the first *S. pyogenes* sequencing project (a serotype M1 strain) was the start in microbial genomics of GAS (Ferretti *et al*., 2001). The University of Oklahoma *S. pyogenes* M1 database was accessible on the Internet from the very beginning of the project and contained regularly updated sequence contigs derived from a shotgun plasmid library. The DNA sequence database could be mined for genes of interest from anywhere in the world long before completion of the project. Although streptococcal and staphylococcal SAgs only share limited amino acid sequence homology (sometimes less than 25%), they all share the two highly conserved "family signature motifs" Y-G-G-[LIV]-T-X(4)-N (Prosite entry PS00277) and K-X(2)-[LIVF]-X(4)-[LIVF]-D-X(3)-R-X(2)-L-X(5)-[LIV]-Y (PS00278). Mining of the *S. pyogenes* M1 genome database with these two motifs resulted in the discovery of four novel *sag* genes, which were designated *speG*, *speH*, *speI*, and *speJ* (Proft *et al*., 1999, 2001). The genes were cloned and expressed in *E. coli*. Functional analysis using recombinant forms of the novel toxins showed that they bound to MHC II and stimulated T cells with high potencies in a Vβ-dependent mode, confirming their predicted role as SAgs. Furthermore, SPE J induced fever in rabbits and was lethal in two rabbit models of STSS (McCormick *et al*., 2001a). In addition, a putative gene fragment encoding a nonfunctional peptide with sequence similarity to the C-terminal region of SPE C was found and named *speK* (Ferretti *et al*., 2001).

Another novel *sag* gene, *speL*, was identified in Japan after DNA analysis of the 41,769 bp temperate phage ΦNIH1.1 from a serotype M3 strain (Ikebe *et al*., 2002). In the same year, the complete *S. pyogenes* M3 genome was analyzed in the U.S. and led to the discovery of the same *sag* gene, which was designated *speK* (Beres *et al*., 2002), a name that had previously been assigned to a pseudogene on the *S. pyogenes* M1 genome (Ferretti *et al*., 2001). In this chapter, we will refer to this SAg as SPE K/L. The *speK/L* gene was also found on a serotype M89 strain from New Zealand and named *speL* in accordance with the Japanese designation (Proft *et al*., 2003). It was identified by PCR analysis with a specific DNA primer pair designed from a novel *sag* gene that was discovered by mining of a *Streptococcus equi* database and named $speL_{Se}$ (see below). The same strategy also led to the discovery of a hitherto unknown *sag* gene in serotype M80 that was designated *speM* (Proft *et al*., 2003b). The SAg nomenclature turned increasingly confusing when another *S. pyogenes* genome (serotype M18) was completed in the U.S. revealing two novel *sag* genes, which were named *speL* and *speM* (Smoot *et al*., 2002a). *SpeL* is identical to *speM* found on the M80 strain and will be referred to here as *speL/M*, while *speM* from the M18 strain has not been reported before. Recombinant forms of SPE K/L and SPEL/M were shown to be potent mitogens targeting T cells in a Vβ-specific and MHC II-dependent mode, which confirmed their role as SAgs (Smoot *et al*., 2002b; Proft *et al*., 2003b).

### *The "false" GAS superantigens*

With the generation of increasingly pure SAg preparation and the use of recombinant proteins, it has become evident that some of the earlier reported mitogenic activities were due to contamination with potent SAgs. For example, mitogenic activity toward T cells carrying the Vβ2, 4, and 8 was found in early preparations of SPE B. Since none of the SAgs known at the time targeted Vβ8 T cells, it was postulated that the activity was caused by traces of a potent unknown SAg, which was called SPE X (Braun *et al*., 1993). Interestingly, Vβ2, 4, and 8 activity was also reported for the so-called mitogenic factor (MF) described by Yutsudo *et al*. (1992)—also designated as SPE F (Norrby-Teglund *et al*., 1994b)—and for pepM5, an M protein-derived peptide from *S. pyogenes* M5 (Tomai *et al*., 1992), while Vβ4 and 8 specific activity was reported in preparations of the *S. pyogenes* mitogen-2 (SPM-2) (Rikiishi *et al*., 1997).

It is now widely believed that the observed superantigenic activities were due to contaminations with SPE C and SMEZ and that SPE X is, in fact, SMEZ (Gerlach *et al*., 1994, 2001; Proft *et al*., 1999; Sriskandan *et al*., 2000).

The gene encoding SPM has never been identified, while SPE F has been shown to be identical to streptococcal DNaseB (Sriskandan *et al*., 2000).

## MOLECULAR BIOLOGY OF STREPTOCOCCAL SUPERANTIGENS

### Genomic location and frequency of GAS superantigen genes

The GAS *sag* genes can be located on the chromosomal backbone or on an integrated bacteriophage region (see Table 51.2), which allows horizontal gene transfer between GAS strains. The first evidence for a mobile GAS *sag* gene was provided by Zabriskie (1964), who showed the conversion of the non-lysogenic $speA^-$ strain $T25_3$ to a $speA^+$ strain by the ϕT12 phage from the *S. pyogenes* strain T12g1. The recent completion of five GAS genome projects has provided further insight into the genomic localization of streptococcal *sag* genes. These included serotypes M1 (Ferretti *et al*., 2001), M3 (Beres *et al*., 2002; Nakagawa *et al*., 2003), M6 (Banks

*et al*., 2004), and M18 (Smoot *et al*., 2002a). Only three of the currently known eleven *sag* genes, *smez*, *speG*, and *speJ*, were found not to be associated with a bacteriophage. Genotyping of 94 clinical GAS isolates from New Zealand showed the *smez* and *speG* genes in 100% of the analyzed isolates, confirming their stable chromosomal location (Proft *et al*., 2000). Interestingly, *speJ* was not found on the M3 and M18 genomes, suggesting that, although not associated with phage, *speJ* might be located on an unstable region on the chromosome.

Comparative genomics revealed that *sag* genes are positioned on a variety of temperate phages and that related phages sometimes harbor different *sag* genes, supporting the idea of recombinational evolution of GAS prophages (Table 51.2) (Ferretti *et al*., 2001; Banks *et al*., 2004).

Four allelic forms of the *speA* gene were identified. *SpeA*1 was found on ϕ8232.1 in *S. pyogenes* MGAS8232 (serotype M18), *speA*3 was found on ϕSPsP2 (strain SSI-1, serotype M3) and on the identical ϕ315.5 (strain MGAS315, serotype M3), while *speA*4 was found on ϕ10394.2 (strain MGAS10394, serotype M6). Apart from the M3 type associated ϕSPsP2 and ϕ315.5, the *speA* containing phages are highly divergent. Instead, ϕ8232.1 (M18) is closely related to ϕ315.1 (M3), which does not contain any *sag* gene.

The *speC* gene was identified on ϕSF370.1 (strain SF370.1, serotype M1) and on the unrelated ϕ8232.2 (M18). Phage 8232.2 (M18) is most closely related to ϕ10394.3 (M6) that carries the *speK/L* gene, while ϕSF370.1 (M1) is related to the M3 type phages ϕ315.4 and ϕSPsP3 (identical to ϕNIH1.1; Ikebe *et al*., 2002), which both also contain the *speK/L* gene. These four prophage elements are inserted at analogous sites, which is the predicted $T12_{att}$ site. Another T12-like phage that inserts at the predicted $T12_{att}$ site is ϕ315.2 (strain MGAS315, M3), which contains the *ssa* gene. Interestingly, the almost identical ϕSPsP5 (strain SSI-1, M3) does not contain *ssa*, nor any other *sag* gene. Instead, in SSI-1, *ssa* is translocated to ϕSPsP6, which is almost identical to ϕ315.1 in MGAS315.

The *speI* and *speH* genes were originally identified in tandem orientation on the defective (non-inducible) phage 370.2 (M1) (Ferretti *et al*., 2001). In addition, a truncated variant of *speI* and a *speH* pseudogene containing 14 internal stop codons were found on ϕ10394.2 (M6), which also contains *speA*4. Phage 370.2 (M1) is most closely related to ϕ8232.3 (M18) that carries the *speL/M* and *speM* genes.

Genotyping of 50 GAS isolates comprising 29 different M types and 3 non-typeable strains has shown *speA*, *speC*, *speH*, *speI*, *ssa*, *speK/L*, and *speL/M* gene frequencies of 30%, 36%, 22%, 12%, 26%, 28%, and 4%, respectively (Proft *et al*., 2003b). Interestingly, only 12% of the isolates contained both *speI* and *speH* (located in tandem orientation on phage 370.2 (M1)). None of the isolates carried the *speI* gene without the *speH* gene. In all the temperate phages analyzed, the *sag* genes were located at the distal end of the integrated phage genome (with respect to the integrase gene), suggesting that these genes may have been acquired (or lost) in the past through some aberrant excision event where host genomic material was acquired by the phage (Ferretti *et al*., 2004). In a study by Ikebe *et al*. (2002), it was shown that none of 10 M3 isolates recovered during or before 1973 carried the *speK/L* gene, whereas all of 18 M3 isolates collected during or after 1992 contained *speK/L*, suggesting a rather recent gene transfer event.

Epidemiological studies have shown associations between *sag* gene frequencies and certain GAS diseases. For example, Yu and Ferretti (1989) showed that among 146 isolates from patients with scarlet fever, 45% were shown to contain *speA*, while only 15% of 300 isolates from patients with various other diseases contained *speA*. In another study, 34 GAS isolates from STSS patients were analyzed and *speA* was found in 85% (Hauser *et al*., 1991). In a study conducted by Reichardt *et al*. (1992), *speA* and *speC* were found in 64%

**TABLE 51.2** Location of the phage-encoded GAS *sag* genes

| SF370 (M1) | | SSI-1 (M3) | | MGAS315 (M3) | | MGAS10394 (M6) | | MGAS8232 (M18) | |
|---|---|---|---|---|---|---|---|---|---|
| phage | *sag* genes | phage | *sag* genes | phage | *sag* genes | phage | *sag* genes | phage | *sag* genes |
| SF370.1 | *speC* | SPsP6 | *ssa* | 315.1 | - | 10394.6 | - | 8232.1 | *speA1* |
| SF370.2 | *speI, speH* | SPsP5 | - | 315.2 | *ssa* | 10394.5 | *speC* | 8232.2 | *speC* |
| SF370.3 | - | SPsP4 | - | 315.3 | - | 10394.4 | - | 8232.3 | *speL/M, speM* |
| SF370.4 | - | SPsP3[a] | *speK/L* | 315.4 | *speK/L* | 10394.3 | *speK/L* | 8232.4 | - |
| | | SPsP2 | *speA3* | 315.5 | *speA3* | 10394.2 | *speA4, speI*[b]*, speH*[b] | 8232.5 | - |
| | | SPsP1 | - | 315.6 | - | 10394.1 | - | | |

[a]identical to ϕNIH1.1
[b]pseudogenes

and 28% of strains from patients with STSS isolated in Europe and Chile.

## Allelic variation in superantigen genes

Allelic variation has been reported for several *sag* genes. However, in most of the cases the divergences were minimal. For example, *speA*1, *speA*2, and *speA*3 encode toxins that differ by a single amino acid (G110S in SPE A 2 and V106I in SPE A 3). In contrast, SPE A4 differs from SPE A-1 by ≈11% and was reported to be frequent in serotype M6 (Nelson *et al*., 1991; Banks *et al*., 2004). *SpeA*5 differs from *speA*4 by four synonymous and one non-synonymous mutation, while *speA*6 differs from *speA*1 by a single synonymous mutation. It has been suggested that *speA* arose from two phylogenetic lineages designated lineage I (*speA*1, *speA*2, *speA*3, *speA*6) and lineage II (*speA*4, *speA*5) (Bessen *et al*., 1999)

Minor allelic variations were also reported for the *speC* gene. The *speC*2 allele differs from *speC*1 by two silent A/G transitions (Kapur *et al*., 1992), while *speC*3, *speC*4, and *speC*5 differ from *speC*2 by one base pair resulting in non-synonymous amino acid changes (Norrby-Teglund *et al*., 1994a). Bessen *et al*. (1999) found a significant non-random association between exotoxin alleles and *emm* patterns. They showed that in 160 GAS isolates, the *speA* and *speC* alleles of organisms harboring the *emm* marker for tissue tropism pattern A–C (pharyngeal reservoir) differed from *spe* alleles that predominate in organisms of pattern D (impetigo). Pattern A–C organisms mainly harbored *speA* lineage I alleles, while pattern D organisms mainly harbored *speA* lineage II alleles. Barriers that prevented horizontal gene transfer were suggested to be responsible for unequal distribution.

Significant allelic variation has also been reported for the *smez* gene. DNA sequence analysis of a *smez* gene cloned from *S. pyogenes* 2035 showed nucleotide changes in 36 positions (=5%) compared to the *smez* gene and was designated *smez-2* (Proft *et al*., 1999). The deduced protein sequence differs in 17 amino acid residues (=8.1%) and the most significant difference is an exchanged pentapeptide sequence at position 96–100, where the EEPMS sequence of SMEZ is converted to KTSIP in SMEZ-2. In a follow-up study, *smez* DNA sequences of 37 GAS isolates were analyzed, representing 22 different M/*emm* types, 5 MNT isolates, and two isolates of unknown M type (Proft *et al*., 2000). As a result, 22 novel *smez* alleles were identified, which appeared to be in linkage equilibrium with the M/*emm* type. Three of the *smez* sequences (*smez-6*, *smez-19*, and *smez-23*) represented pseudogenes that have single base pair deletions causing frame shifts. Interestingly, the *smez* gene shows a significant mosaic structure, suggesting that the polymorphism has arisen from homologous recombination events rather than random point mutations. The biological function of SMEZ, e.g., binding to MHC class II and Vβ-specific stimulation of T cells, was not affected by the allelic variation. However, the SMEZ variants showed antigenic differences. Neutralization responses of individual human sera from healthy donors against different SMEZ variants varied significantly.

## Regulation of superantigen production

The production of SPE A and SPE C in GAS cell culture supernatant was shown to differ significantly between individual isolates. Eleven strains from various M/T serotypes were shown to produce between 0.03 and 16 mg/ml, while the amounts of SPE C produced by three different strains were 0.9, 1.2, and 1 mg/ml (Gerlach *et al*., 1981). Higher amounts of toxin were produced by 26 isolates from patients with STSS, with average concentrations of 3.2 mg/ml of SPE A and 0.6 mg/ml of SPE C (Lee and Schlievert, 1989). In contrast, many strains isolated from patients with STSS in France, Sweden, and Chile produced very low or no detectable amounts of both toxins (Köhler, 1990; Reichardt *et al*., 1992). It has been suggested that *speA* expression varies in a clonal manner with respect to the M serotype (Kim and Schlievert, 1997). However, several recently conducted experiments have put a different light on this topic. It now seems that *sag* gene expression is tightly controlled and that host factors might be involved in the up-regulation of SAgs during infection. Broudy *et al*. (2001) reported the up-regulation of SPE C in GAS that were co-cultured with human pharyngeal cells. This also led to the induction of the bacteriophage that harbors the *speC* gene, and the induction event was mediated by a soluble factor produced by the pharyngeal cells. Co-culture of strain MGAS315 with Detroit 562 (D562) human epithelial pharyngeal cells induced the prophage encoding *speK/L* (ϕ315.4). However, no significant production of SPE K/L was observed (Banks *et al*., 2003). Surprisingly, the production of SPE A increased during co-culture of GAS with D562 cells despite the lack of induction of the prophage encoding *speA* (ϕ315.5).

Using a mouse infection chamber model, Kazmi *et al*. (2001) monitored *speA* gene induction *in vivo* at various time points after infection. They implanted Micropore Teflon diffusion chambers subcutaneously in BALB/c mice and inoculated the chambers with clonal M1T1 isolates expressing no detectable amounts of SPE A *in vitro*. After seven days postinfection, SPE A expression was detected in the chamber fluid of all animals tested. Interestingly, isolates recovered from the chamber and

grown *in vitro* continued to produce SPE A even after 21 passages, suggesting a stable switch on the *speA* gene.

The molecular mechanisms of *sag* gene regulation are only poorly understood. However, a recent study has shown that the *speA* gene and several other virulence genes are under the control of the Nra transcription regulator, a member of the RofA-like protein (RALP) family (Podbielski *et al*., 1999). Nra represses the expression of several virulence genes, including *speA*. Maximum expression of Nra occurs in the early stationary growth phase, but does not appear to respond to changing atmospheric conditions.

## SUPERANTIGENS FROM NON-GAS

Group C streptococcus (GCS) and group G streptococcus (GGS) are commonly regarded as commensals usually found in association with the normal flora of human skin, pharynx, and intestine. Recently, there have been an increasing number of reports implicating GCS and GGS with severe invasive infections, such as necrotizing fasciitis and toxic shock syndrome (Oster and Bisno, 2000). Although mitogenic activity in supernatant of clinical GCS and GGS isolates had been reported over several years, superantigens had not been identified until recently. The first report came from Timoney's group who identified two superantigens in *Streptococcus equi*, a bacterium that causes strangles in horses, but can also infect humans. The *Streptococcus equi* pyrogenic exotoxins (SePE)H, I, are highly homologous to their *S. pyogenes* counterparts SPE H and SPE I (greater than 98% amino acid sequence identities), indicating horizontal gene transfer from *S. pyogenes* to *S. equi* or vice versa (Artiushin *et al*., 2002). Similar to GAS, *sepe-I* and *sepe-H* were found in tandem orientation at the distal end of an integrated phage genome. Interestingly, both genes were not found in the closely related *Streptococcus zooepidemicus*, and it was suggested that the acquisition of the *sag* genes might be an important event in the formation of a more virulent *S. equi* strain from its putative *S. zooepidemicus* ancestor (Artiushin *et al*., 2002). SePE I and SePE H both elicited strong mitogenic responses from horse peripheral blood mononuclear cells. Moreover, both toxins were pyrogenic for rabbits, but only SePE I was pyrogenic in ponies. Horses recovered from strangles or immunized with SePE I were seropositive and resistant to the pyrogenic effects of SePE I, suggesting a potential role for this toxin in strangles.

Another two *sag* genes were identified by data mining of the *S. equi* genome at the Sanger Centre and named $speL_{Se}$ and $speM_{Se}$ due to the homology to their *S. pyogenes* counterparts, *speL* and *speM* (here described as *speK/L* and *speL/M*) with 99% and 98.1% nucleotide identities, respectively (Proft *et al*., 2003b). Interestingly, both genes were not detected in eight genetically different *S. equi* isolates, suggesting that, like their GAS counterparts, these genes are also rare in GCS isolates.

Four SAgs have been identified from *Streptococcus dysgalactiae* subsp. *equisimilis*. All of them are highly similar to their GAS counterparts SPE A, SPE C, SPE G, and SPE M. *Streptococcus dysgalactiae*-derived mitogen (SDM) is 99% similar to SPE M (Miyoshi-Akiyama *et al*., 2003), and SPE $G^{dys}$ is 86% similar to SPE G (Sachse *et al*., 2002). Kalia and Bessen (2003) analyzed 34 genetically distinct group C/G streptococci and found the *speA* gene in three isolates and *speC* in one isolate. The *speA* alleles from two isolates were identical to GAS alleles *speA*2 and *speA*4, respectively, while the third *speA* allele is characterized by two unique nucleotide substitutions that are not observed among *speA* alleles from GAS. This was designated *speA*7. However, the translated protein is 100% identical to the SPE A5 variant of GAS. The *speC* allele was found to be identical to *speC*1 of GAS.

In all non-GAS toxins, amino acid exchanges are outside the MHC class II and TcR binding sites, suggesting that the GAS toxins and the non-GAS toxins are orthologues with identical functions. A recent study has shown that GAS, GCS, and GGS exchange housekeeping genes by horizontal gene transfer with strong net directionality of gene movement from GAS donors to GCS and GGS recipients (Kalia *et al*., 2001). The fact that all GAS counterparts of GCS/GGS *sag* genes, except *speG*, were found on prophages and the strong homology between the orthologues suggests that commensal GCS/GGS strains might have acquired these *sag* genes from pathogen GAS generating more virulent GCS and GGS.

## BIOCHEMICAL PROPERTIES OF SUPERANTIGENS

### Binding to MHC class II

All SAs bind to MHC class II as a prerequisite for T cell recognition. The molecular mechanism of this interaction is not only different from the binding of conventional peptide antigens, but also significantly varies between individual SAgs. In general, SAgs can be divided into three groups according to their binding mode to MHC class II; (i) SAgs that bind to the invariant MHC class II α-chain only; (ii) SAgs that bind to the polymorphic MHC class II β-chain only; and (iii) SAgs that bind to both MHC class II chains.

### *Streptococcal SAgs that bind to the MHC class II β-chain*

With the exception of SPE A and SSA, all streptococcal SAgs appear to bind to the MHC class II β-chain only. The first evidence for this mode of binding was provided for the staphylococcal enterotoxin A (SEA), when Herman *et al*. (1991) showed that SEA binds effectively to most HLA-DR alleles and isotypes, such as DR1, but only poorly to DRw53. The difference in binding was localized to a single amino acid residue at position 81 in the beta 1 domain, a highly conserved histidine that is replaced by a tyrosine in DRw53. It was later shown that H is 81 is part of a zinc coordination that also involves SEA residues His187, His225, and Asp227 (Hudson *et al*., 1995). The relative binding affinity of this interaction is about 100-times higher than the generic low-affinity site ($10^{-7}$ M) that SEA and several other SAgs use for binding to the MHC class II α-chain and has therefore been referred to as the "high-affinity binding site." The first streptococcal SAg shown to possess this mode of binding was SPE C. Li *et al*. (1997) reported that SPE C binding to MHC class II was completely abolished in the presence of EDTA, but could be restored by excess of $Zn^{2+}$ over EDTA. Moreover, SPE C did not prevent binding to MHC class II of two typical MHC class II α-chain binding SAgs from *S.aureus*, staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin (TSST). Structural analysis of SPE C revealed a zinc-binding site in the C-terminal domain, similar to that in SEA involving SPE C residues His167, His201, and Asp203 (Roussel *et al*., 1997). The complete zinc coordinated binding was finally confirmed with the successful co-crystallization of SPE C with HLA-DR2a bearing a peptide derived from myelin basic protein (MBP). The structure not only showed the conserved tetrameric zinc complex, but also revealed extensive interaction of the bound peptide, such that the peptide accounts for approximately one-third of the surface area of the MHC class II molecule buried in the complex (Li *et al*., 2001; Petersson *et al*., 2001). This is comparable to the contribution of the antigenic peptide in the TcR-MHC complexes. SPE C makes several conserved interactions with bound peptide, in particular Gln113 of SPE C, which forms a hydrogen bond to the backbone of the P3 residue of the peptide. Certain other interactions involving peptide side chains are unique to the bound peptide, suggesting a wide range of affinities for SAg-binding to the MHC class II peptide complex. It has been suggested that SPE C may optimize T cell responses by mimicing the peptide dependence of conventional antigen presentation and recognition (Li *et al*., 2001).

Crystal structures and computer-generated models of SPE G, SPE H, SPE I, SPE J, SPE K/L, SPE L/M, SPE M, and SMEZ showed the conserved zinc-binding site in the C-terminal domain of all of these proteins. Zinc dependency was demonstrated by biochemical analysis: When binding to MHC class II was completely abolished in the presence of EDTA, but could be restored by excess of $Zn^{2+}$ over EDTA (Proft *et al*., 1999, 2001). Furthermore, these toxins lack the generic MHC class II α-chain binding motif (a hydrophobic loop region in the N-terminal domain) and did not prevent SEB and TSST from binding to MHC class II in competition experiments (Proft *et al*., 1999, 2001, 2003b). On the other hand, Scatchard blot analysis of SPE G, SPE H, SMEZ-1, and SMEZ-2 revealed two distinct binding affinities: a high affinity binding of 16–65 nM, similar to the zinc-dependent MHC class II β-chain binding of SEA, and a low-affinity binding of 1–2 μM. Moreover, these toxins only partially competed against each other for MHC class II binding with a clear hierarchical order [(SPE C, SMEZ-1) > SMEZ-2 > SPE H > SPE G], where SPE G did not compete with any other toxin. Due to the lack of any potential MHC class II α-chain binding motifs in the toxin sequences, it was suggested that some toxins have a more restricted repertoire of MHC class II molecules defined by the bound antigenic peptide and that the multiphasic Scatchard blots resulted from a variety of affinities ranging from nanomolar to micromolar defined by the bound peptide (Proft *et al*., 1999). Further evidence was provided when the SPE C – HLA-DR2a structure revealed extensive interaction of the bound peptide (Li *et al*., 2001). This raises the possibility that certain bound peptides could enhance the potency of the SAg by promoting high-affinity but low-density binding to MHC class II (Wen *et al*.,1996; Hogan *et al*., 2001). This mechanism might be important to avoid T cell apoptosis through supra-optimal signaling caused by high ligand densities.

### *Streptococcal SAgs that bind to the MHC class II α-chain*

Only two streptococcal SAgs, SPE A and SSA, lack the C-terminal zinc-binding site and are therefore unable to bind to the MHC class II β-chain. Both SAgs bind to the generic MHC class II α-chain, predominantly to HLA-DQ, rather than HLA-DR1 molecules.

The overall structure of SPE A is more closely related to the staphylococcal enterotoxins B and C (SEB, SEC) than to any other streptococcal SAg (Papageorgiou *et al*., 1999). It has been shown that SPE A competes with SEB for binding to HLA-DR1 molecules, suggesting that both toxins have a common recognition site for MHC class II (Hartwig *et al*., 1994). SEB is a prototype SAg for MHC class II α-chain binding. A struc-

tural study of SEB complexed with HLA-DR has shown that this toxin possesses an exposed hydrophobic loop region within the N-terminal β-barrel domain to bind to a hydrophobic groove located in the distal region of the DR α1 domain with binding affinities of $10^{-5}$ M (Jardetzky *et al*., 1994). This interaction has been referred to as the "generic" or the "low-affinity" binding site. In addition, SEB binds to a protruding loop of the DR1 α-chain using a hydrophilic pocket on the SEB surface created by Glu67, Tyr89, and Tyr115. However, there are differences in binding affinity for MHC class II. SPE A has a greater affinity for HLA-DQ than it does for HLA-DR or HLA-DP, while SEB has a greater affinity for HLA-DR, suggesting that SPE A and SEB have similar but not identical MHC class II binding sites. Lys39 from HLA-DR1, a key residue present in all three allotypes, forms a salt bridge with Glu67 of SEB, and this residue is conserved in SPE A (Glu61), suggesting that SPE A might also target Lys39 on the MHC class II α-chain, but in a binding conformation different from SEB binding (Papageorgiou *et al*., 1999). In another study, alanine substitutions of each of the SPE A residues Leu42, Asp45, Leu46, Ile47, Tyr48, and Tyr83 resulted in significant decrease in affinity for HLA-DQ (Kline and Collins, 1996). With the exception of Leu42 and Tyr83, these residues are conserved in SEB, but do not appear to be involved in MHC class II binding. Hence, these residues might form a binding interface specific to SPE A. Structural analysis of SPE A has also revealed a zinc-binding site involving residues Asp77, His106, and His110 (Papageorgiou *et al*., 1999). This site is structurally equivalent to the zinc-binding site in SEC2, but different from the "classical" zinc-binding site of the other streptococcal SAgs. From structural modeling of the SPE A -HLA-DR interaction, a potential zinc-dependent binding to the MHC class II β-chain has been suggested, but this hypothesis has not yet been experimentally confirmed (Baker *et al*., 2001).

SSA is structurally closer related to SEB. Structural modeling showed that the SEB residues Glu67, Tyr89, and Tyr115 of the hydrophilic pocket are conserved in SSA (Glu67, Tyr89, and Tyr110). Furthermore, presentation by an HLA-DR1α K39A mutant decreased SSA-induced T cell proliferation by two to three log-fold (Stevens, K. *et al*., 1996). However, the mitogenicity of each of the three SSA mutants, E67A, Y89A, and Y110A, was only slightly decreased relative to SSA wild-type when presented by HLA-DR1 expressing cells. Moreover, competition-binding studies showed that SEB and SSA did not cross-compete, despite effectively competing for DR1 binding and despite the common involvement of DR1α Lys39 (Stevens, K. *et al*., 1996). This lack of competition was later shown to be due to the preference of SSA for HLA-DQ molecules. Five residues were implicated in conferring HLA-DQ binding specificity to SSA as compared to SEB. These included Gln46, His47, Lys65, Asn69, and Asn92 (Sundberg and Jardetzky, 1999).

### *Binding to the T cell receptor*

Until recently, little was known about how SAgs interact with TcR molecules, mainly due to the lack of suitable amounts of recombinant TcR protein for crystallization. In 2002, two complexes were determined—that of SPE A bound to mouse Vβ8.2 and of SPE C bound to human TcR Vβ2.1 (Sundberg *et al*., 2002a). The SPE A-mVβ 8.2 complex is quite similar to the previously reported SEB-mVβ8.2 complex, which is probably not much of a surprise considering the structural homology between SPE A and SEB. The TcR binding site is located in a shallow groove between the two globular domains of the SPE A molecule created by residues from the α2 helix, the β2-β3 loop, the β4 strand, the β4-β5 loop, the β5 strand, and the α5 helix. In the mTcR Vβ8.2 molecule, residues from the complementary-determining region 2 (CDR2), framework region 2 (FR2), and, to a lesser extent, hypervariable region 4 (HV4) and FR3 were found to be involved in SAg recognition. In contrast to the SEB-mVβ8.2 complex, SPE A also interacts with the mVβ8.2 CDR1 loop via a hydrogen bond between Glu94 on the disulfide loop region of SPE A and Asn28 on the mVβ8.2 CDR1 loop. The positionally equivalent residue in SEB (Thr99) is unable to form an intermolecular contact with the CDR1 loop due to differences in side chain length. There are also a number of other hydrogen bonds that involve side chain atoms of both SPE A and mVβ8.2, which is in sharp contrast to the exclusive use of main chain contacts in the SEB-mVβ8.2 complex (Li *et al*., 1998). Extensive mutational analysis of SPE A has shown that eight mutant toxins (S13A, N17A, N20A, D45A, I47A, Y55A, Y84A, and L198A) showed an intermediate capability to stimulate human Vβ2.1-positive cells, while amino acid substitutions at five positions (L24A, Y55A, C90S, C98S, and Q194A) resulted in decreased ability to stimulate human Vβ12.2 expressing cells, and twelve mutant toxins (N17A, N20A, L24A, V30A, N54A, Y55A, Y83A, Y84A, C90S, C98S, Q194A, and L198A) showed a reduced capability to stimulate human Vβ14.1-positive cells (Kline and Collins, 1997). Compared to the SPE A-mVβ8.2 complex, only five of the mutated residues (Asn17, Asn20, Asn54, Tyr84, and Gln194) make contact with the mouse Vβ8.2 region, and there is no correlation between the effect of specific mutations and the SPE A residues involved in binding mVβ8.2. Thus, it is most probable that the SPE A-mVβ8.2 complex shows a binding mode that is distinct from the binding of SPE A to other Vβ regions.

The interaction between SPE C and hVβ2.1 is different altogether and includes a significantly larger buried surface area than the other complexes. The number of intermolecular contacts is higher and also includes the highly variable hVβ2.1 CDR3 loop. Interestingly, SPE C displays a much higher specificity to stimulate T cells than SEB or SPE A, targeting primarily the hVβ2.1 TcR. It has been proposed that this specificity derives from two single amino acid insertions, one each in CDR1 and CDR2, and an extended CDR3 loop. The CDR1 insertion shifts this loop towards the SPE C molecule, resulting in additional intermolecular contacts involving hydrogen bonds with SPE C residues Arg45, Tyr49, and Asn79, while the CDR2 insertion produces a non-canonical CDR2 loop that positions the inserted residue for optimal contact with SPE C. The CDR2 contact region includes hydrogen bonds with SPE C residues Tyr15, Glu178, and Arg181. Mutational analysis confirmed the crucial role of Tyr15 in TcR binding when the Y15A mutant was shown to decrease mitogenicity by nearly 1,000-fold without affecting MHC class II binding (Yamaoka *et al*., 1998).

Recently, the crystal structure of SPE J has been resolved (Baker *et al*., 2004). SPE J is structurally most closely related to SPE C and also primarily targets T cells carrying the hVβ 2.1 TcR. Mutational analysis of SPE J revealed Tyr14 and Arg181 (corresponding to SPE C residues Tyr15 and Arg181, respectively) are key residues for TcR binding. Both SPE J mutants, Y14A and R181A, decreased T cell mitogenicity by 10,000-fold compared to wild-type. In addition, SPE J mutant F77A reduced potency by 100-fold, although like the corresponding residue in SPE C (Leu78), it can only form van-der-Waals interactions. The neighboring residue in SPE C (Asn79) is hydrogen bonded to TcR, but mutation in the equivalent SPE J residue (T78A) had only little effect. Interestingly, SPE C residue Tyr49, which forms hydrogen bonds with the CDR1 loop, is replaced by Phe48 in SPE J and can only form van-der-Waals interactions. Furthermore, an F48A mutant did not result in a detectable decrease in mitogenicity. Thus, despite the structural similarity between SPE C and SPE J and the common preference for the hVβ2.1 TcR, there appears to be a certain degree of variation in the TcR binding mode.

Based on structural observations, three categories of SAg-TcR complexes have been proposed: (i) highly promiscuous T cell activators, including SEB and SEC3, that bind TcR β chains in a simple conformation-dependent manner and interact only with a single CDR loop, CDR2; (ii) moderately promiscuous T cell activators, including SPE A, that form direct side chain/side chain contacts overlayed onto the conformation dependence of the first group, and the additional involvement of the CDR1 loop; (iii) highly selective T cell activators, including SPE C (and probably SPE J), that bind to TcRVβ domains with the highest degree of structural dissimilarity, and the usage of all three CDR loops (Sundberg *et al*., 2002b).

## Dimer-formation of streptococcal superantigens

SPE C was the first streptococcal SAg shown to exist in both monomeric and dimeric forms at neutral or alkaline pH (Li *et al*., 1997). The results were supported by the SPE C crystal structure, which showed that the surface that forms the generic MHC class II α-chain binding site in several other superantigens is used to create a SPE C dimer (Roussel *et al*., 1997). Dimerization appears to be stabilized by an additional zinc-binding site (Zn-B) in which $Zn^{2+}$ is complexed by four SPE C residues, His35, and Glu54 from each of the two monomers. However, zinc does not appear to be essential as dimerization is not affected by EDTA. The biological function of SPE C dimer formation is unknown, but it has been suggested that it might lead to cross-linking of MHC class II molecules on the surface of antigen-presenting cells and that this may have a stimulatory effect on the antigen-presenting cells (Roussel *et al*., 1997). Such an effect has been demonstrated previously for SEA (Tiedemann and Fraser, 1996). MHC class II cross-linking by SEA (a bivalent staphylococcal SAg that binds to both MHC class II chains) resulted in rapid induction of homotypic B cell aggregation, tyrosine kinase activation, and proinflammatory cytokine gene expression. Homotypic B cell aggregation has also been demonstrated for SPE C (Li *et al*., 1997).

The recently resolved crystal structure of SPE J revealed a different mode of homodimerization (Baker *et al*., 2004). SPE J uses the same interface that is used for TcR binding in other SAgs. Site-directed mutagenesis showed that this face is also used for TcR binding in SPE J, and it was implicated that SPE J may have dual functions—cross-linking of MHC class II and TcR as a monomer and cross-linking of two MHC class II molecules as a dimer.

A similar mechanism has recently been demonstrated for SSA (De Marzi *et al*., 2004). Monomeric and dimeric forms of SSA were found in supernatants of GAS cultures. Dimer formation occurred via an intermolecular cysteine bridge involving Cys26 and, like in the SPE J dimer, most probably occludes the TcR interaction site. Intermolecular cysteine bridge formation is also responsible for homodimerization of SPE K/L (Proft, unpublished data).

These observations suggest that at least some streptococcal SAgs might have other function(s) in addition to T cell mitogenicity.

## BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES

### Potency and Vβ-specificity

The potency of SAgs is generally described as half-maximum response ($P_{50}$), which is the amount of toxin needed to achieve 50% of the maximum T cell proliferation. Within the streptococcal SAgs, $P_{50}$ values range between 0.02 pg/ml (SMEZ-2) and 50 pg/ml (SPE H). SMEZ-2 is the most potent of all known SAgs and recombinant SMEZ-2 was shown to be still active at less than 0.1 fg/ml on human peripheral blood lymphocytes (Proft *et al*., 1999). Streptococcal SAgs also cause T cell proliferations in certain animals, such as rabbits and mice. The $P_{50}$ values, however, are usually significantly higher.

A hallmark of SAgs is their specificity for particular TcRVβ regions (see Table 51.1). Considering the fact that the human genome codes for 50 different Vβ regions of which around 20 are commonly expressed, it seems rather interesting that the streptococcal SAgs appear to possess a significant preference for binding certain Vβs. For example, SPE C and SPE J primarily target T cells carrying the Vβ2 TcR, a target that is also used by SPE A, SPE G, SPE H, and SMEZ. Another common target are T cells carrying the Vβ1 TcR, which are primarily stimulated by SPE K/L, SPE M/L, and SPE M (Smoot *et al*., 2002b; Proft *et al*., 2003b), and less frequently by SSA. The reason for the preferential stimulation of certain T cell subpopulations remains a mystery.

### Pyrogenicity and augmentation of endotoxin activity

Pyrogenicity, or the induction of fever, is believed to be a common feature of all streptococcal superantigens, although this has not been experimentally confirmed for all toxins. The IV injection of rabbits with either SPE A or SPE C resulted in elevated temperatures up to 4–5 h after a 30–60 min latency as reported by Schuh *et al*. (1970). The minimal pyrogenic dose of SPE A and SPE C determined 3–4 h after injection ranged between 0.1 and 0.7 μg/kg. The ability to cause fever in rabbits has also been reported for SPE J (McCormick *et al*., 2001a) and for SMEZ (Müller-Alouf *et al*., 2001). It has been suggested that pyrogenicity is due to SAg-induced release of IL-1 and TNF-α by macrophages and the effect of these cytokines on the hypothalamus (Fast *et al*., 1989).

Streptococcal SAgs can enhance the host's susceptibility to lethal endotoxic shock. Rabbits that received SPE C alone showed fevers only, but those given both SPE C and endotoxin showed initial fever followed by hypothermia, labored breathing, diarrhea, evidence of vascular collapse, and finally death (Schlievert, 1982). Susceptibility of rabbits to lethal shock by endotoxin was enhanced by as much as 50,000-fold. It has been suggested that endotoxin and superantigen might act synergistically as a double-hit, resulting in toxic shock in the absence of a typical focus of bacterial infection (Llewelyn and Cohen, 2002).

### Cytokine induction

Superantigens are potent inducers of cytokines. Toxin-mediated activation of T cells results in a rapid systemic release of TNF-α and TNF-β, followed sequentially by IL-2, IL-6, IL-1, and IFN-γ (Jupin *et al*., 1988; Miethke *et al*., 1992; Hackett and Stevens, 1993). In particular, TNF-α increases dramatically within the first few hours of SAg stimulation as demonstrated in animal studies. TNF-α induces the production of IL-1 and IL-6 and acts synergistically with IL-1 to cause fever and shock. Besides these pro-inflammatory cytokines, the release of anti-inflammatory cytokines, such as IL-4, IL-5, IL-10, and IL-1 RA, and the production of hematopoietic cytokines, such as IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) after stimulation with SPE A or SPE C, has also been reported (Müller-Alouf *et al*., 1996, 1997; Rink *et al*., 1996). A comparative study with SPE A and SMEZ revealed that both toxins elicited the release of substantial amounts of pro- and anti-inflammatory, chemotactic, and hematopoitic cytokines. However, the cytokine-inducing capacity of SMEZ was approximately 10-fold higher than that of SPE A (Müller-Alouf *et al*., 2001). Furthermore, disruption of the *smez* gene in an M89 GAS strain abrogated the immunoactive properties of the strain completely. Despite the capability to produce several other SAgs, supernatant from the *smez*-mutant was unable to elicit production of IFN-γ, TNF-α, TNF-β, IL1β, or IL8 from PBMCs, suggesting that SMEZ might be the major immunoactive SAg produced by GAS (Unnikrishnan *et al*., 2002).

## SUPERANTIGENS AND HUMAN DISEASE

### Invasive GAS disease and STSS

GAS is associated with a number of severe invasive diseases, including cellulitis, myositis, and necrotizing fasciitis (or flesh-eating disease) (Stevens, 1992, 2000; McCormick *et al*., 2001b; Seal, 2001). The most severe form of invasive GAS disease is STSS with mortality rates of 30–70%.

The clinical symptoms are very similar to those in staphylococcal toxic shock, but STSS is often associated with bacteremia, myositis, or necrotizing fasciitis. STSS is characterized by rash, hypotension, and multiple organ failure.

Several lines of epidemiological, experimental, and clinical observations strongly imply that at least some SAgs play a pivotal role in the pathogenesis of STSS and possibly in other invasive GAS diseases.

### *Epidemiological studies*

Serotype M1 and M3 strains have been predominantly isolated from patients with STSS. Both strains frequently produce SPE A and SPE C (Yu and Ferretti, 1989). The *speA* gene was found in a majority (40–90%) of GAS isolates from the USA associated with invasive GAS disease and STSS, but only in a minority (15–20%) of isolates from non-invasive diseases like pharyngitis (Yu and Ferretti, 1989; Hauser *et al*., 1991). A high frequency of *speA* (80%) was also observed in isolates from STSS patients collected in Australia (Carapetis *et al*., 1995). A study by Reichardt *et al*. (1992) of 53 strains from patients with STSS from Europe and Chile found *speA* and *speC* in 64% and 28%, respectively. In contrast, a more recent study by Hsueh *et al*. (1998) showed no significant difference in the frequency of *speA* between invasive and non-invasive GAS isolates collected in Taiwan (39% and 36%, respectively). Furthermore, among the invasive strains, *speA* was found in only 18% of the isolates associated with STSS cases. The recent discovery of several novel streptococcal *sag* genes, with *speG* and *smez* present on all strains analyzed thus far, might explain the development of STSS in these cases. In addition, regulation of *sag* gene expression appears to play an important role (see above).

### *Animal infection models*

Using a baboon model of GAS bacteremia that mimics human STSS, Stevens, D.L. *et al*. (1996) showed that intravenous infusion of a SPE A-producing serotype M3 strain resulted in profound hypotension, leukopenia, metabolic acidosis, renal impairment, thrombocytopenia, and disseminated coagulopathy within three hours. Serum TNF-α peaked at three hours and anti-TNF-α antibodies markedly improved arterial pressure and survival, indicating the important role of TNF-α in STSS.

Experiments with isogenic GAS strains lacking the *speA* gene administered into rabbits showed that only the wild-type strain caused STSS, while the *speA*-knockout strain did not (Schlievert *et al*., 1996). Sriskandan *et al*. (1996a) used an isogenic *speA*$^-$strain to evaluate the role of SPE A in a murine model of bacteremia and streptococcal muscle infection. Surprisingly, disruption of *speA* was not associated with attenuation of virulence and paradoxically the *speA*$^-$ mutant led to increased bacteremia and a reduction of neutrophils at the site of primary muscle infection. A possible explanation for this unexpected result is that SAgs are significantly less active on murine PBLs (Proft *et al*., 1999), probably due to inefficient binding to murine MHC class II. Indeed, different results were obtained in studies with HLA-DQ transgenic mice (Welcher *et al*., 2002; Sriskandan *et al*., 2001). Expression of HLA-DQ rendered the mice susceptible to SPE A-induced lethal shock that was accompanied by massive cytokine production. Immune activation during GAS infection was manifested by Vβ-specific T cell repertoire changes and widespread lymphoblastic tissue infiltration. In contrast, lymphoid activation was undetectable in transgenic mice infected with an isogenic *speA*$^-$ strain, demonstrating the pivotal role of a single SAg in pathogenesis of invasive GAS disease.

*In vivo* experiments using an i.p. model of infection demonstrated that SMEZ did not contribute to mortality or impede bacterial clearance in HLA-DQ transgenic mice, but led to a rise of Vβ11 T cells in the spleen (Unnikrishnan *et al*., 2002). Interestingly, infection with an isogenic M89 *smez*$^-$ strain failed to elicit a significant cytokine production compared with the parent strain, but resulted in a clear rise in murine Vβ4 T cells, suggesting a role for SMEZ as a repressor of cognate anti-streptococcal response.

### *Clinical studies*

SPE A has been detected by ELISA in the sera of STSS patients, and its presence was associated with elevated levels of TNF-β, providing some evidence of SPE A-induced T cell activation (Sriskandan *et al*., 1996b). In a more recent study, strong mitogenic activities were observed in the serum of two patients with STSS, one of whom died. Although the infecting GAS strains carried several *sag* genes, including *speA*, *speC*, *speG*, *speJ*, and *smez*, the mitogenic activity could be wholly attributed to SMEZ, with a small contribution of SPE J in one case (Proft *et al*., 2003a). The quantity of circulating toxin was estimated to be in the order of 100pg/ml. The surviving patient developed neutralizing antibodies against SMEZ during convalescence, providing further evidence for the importance of SMEZ in STSS. Furthermore, the results suggested that the lack of neutralizing antibodies against SAg might be a risk factor for invasive GAS disease. Supportive evidence also came from other studies. Eriksson *et al*. (1999) reported that neutralization of SPE A lymphocyte mitogenicity was totally absent in sera from patients with STSS and

low in sera from patients with uncomplicated bacteremia, compared with levels in sera from uncomplicated erysipelas. Another study showed that the levels of neutralizing anti-SPE A antibodies in plasma samples from severe and non-severe invasive GAS infections were significantly lower than in age- and geographically matched healthy controls (Basma *et al*., 1999).

### *Genetic background of the host*

Recent results suggested that the genetic background might influence the susceptibility for invasive GAS disease. In particular, HLA polymorphism might play a role in disease susceptibility. Evidence was provided by Kotb and coworkers, who found that SPE A triggered significantly higher proliferative responses when presented by HLA-DQ compared to HLA-DR1, -DR4, or -DR5 alleles. In contrast, SPE C was preferentially presented by DR4 (Norrby-Teglund, 2002). Moreover, patients with the DRB1$^{*}$1501/DQB1$^{*}$0602 haplotype mounted significantly reduced responses to streptococcal SAgs and were less likely to develop severe systemic disease compared to individuals with risk or neutral haplotypes (Kotb *et al*., 2002). The dependence of SPE A on HLA-DQ α-chain polymorphism was demonstrated by Llewelyn *et al*. (2004), who showed that SPE A binds more strongly to HLA-DQA1*01 than to HLA-DQA1*03/05 and that differential HLA-DQ binding results in quantitative and qualitative differences in T cell proliferation, cytokine production, and Vβ-specific changes in the T cell repertoire.

## Acute rheumatic fever (ARF)

ARF is a postinfection sequelae and the leading cause of preventable pediatric heart disease. It usually occurs in school-age children and young adults after pharyngeal infection with *S. pyogenes*. ARF is a cross-reactive immune response to the host's cardiac tissue, and it has been proposed that the reactive T cells might be driven by SAgs. Recently, several novel streptococcal SAgs have been identified from ARF-associated serotypes. The genes for SPE K/L were found in high frequencies on serotypes M3 (USA and Japan) (Beres *et al*., 2002; Ikebe *et al*., 2002) and on M89 (New Zealand) (Proft *et al*., 2003b), while SPE L/M and SPE M were found in M18 (USA) (Smoot *et al*., 2002a). It was shown that antibodies against SPE L/M and SPE M were more common in convalescent sera from ARF patients compared to patients with pharyngitis (Smoot *et al*., 2002b). Interestingly, a common target of the SAgs SPE K/L, SPE L/M, and SPE M are T cells bearing the TcRs with Vβ1.1.

## Kawasaki disease (KD)

KD is an acute multi-system vasculitis of unknown etiology that affects mostly young children and is now recognized as the leading cause of acquired heart disease in children in the developed world. Although KD has been reported all over the world, it is overexpressed among Asian populations, especially Japanese (Cimaz and Falcini, 2003).

KD is associated with marked activation of T cells and monocytes, and there is a remarkable similarity among KD, staphylococcal toxic shock, STSS, and scarlet fever in the clinical symptoms. Intravenous immunoglobulin therapy is highly effective when given early, suggesting that the causative agent is a toxin. Several investigators reported the selective expansion of T cells bearing the Vβ2.1 TcR, which points towards a SAg involvement in the disease (Abe *et al*., 1992; Konishi *et al*., 1996). Elevated plasma levels of IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNF-α were observed in the acute phase of KD and levels of anti-SPE C antibodies were significantly higher in patients with acute and convalescent KD than in age-matched controls (Yoshioka *et al*., 1999).

## ACKNOWLEDGMENTS

The authors are supported by the Health Research Council New Zealand.

## REFERENCES

Abe, J., Forrester, J., Nakahara, T., Lafferty, J., Kotzin, B. and Leung, D. (1991). Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B. J. *Immunol*. **146,** 3747–3750.

Abe, J., Kotzin, B.L., Jujo, K., Melish, M.E., Glode, M.P., Kohsaka, T. and Leung, D.Y. (1992). Selective expansion of T cells expressing T cell receptor variable regions V beta 2 and V beta 8 in Kawasaki disease. *Proc. Natl. Acad. Sci. USA* **89,** 4066–4070.

Artiushin, S.C., Timoney, J.F., Sheoran, A.S. and Muthupalani, S.K. (2002). Characterization and immunogenecity of pyrogenic mitogens SePE-H and SePE-I of *Streptococcus equi*. *Microbial. Path*. **32,** 71–85.

Baker, H., Proft, T., Webb, P., Arcus, V., Fraser, J. and Baker, E. (2004). Crystallographic and mutational data show that the streptococcal pyrogenic exotoxin J can use a common binding surface for T cell receptor binding and dimerization. *J. Biol. Chem*. **279,** 38571–38576.

Baker, M., Gutman, D., Papageorgiou, A., Collins, C. and Acharya, K. (2001). Structural features of a zinc binding site in the superantigen strepococcal pyrogenic exotoxin A (SpeA1): implications for MHC class II recognition. *Protein Sci*. **10,** 1268–1273.

Banks, D., Lei, B. and Musser, J. (2003). Prophage induction and expression of prophage-encoded virulence factors in group A Streptococcus serotype M3 strain MGAS315. *Infect. Immun*. **71,** 7079–7086.

Banks, D., Porcella, S., Barbian, K., Beres, S., Philips, L., Voyich, J., DeLeo, F., Martin, J., Somerville, G. and Musser, J. (2004). Progress toward characterization of the group A Streptococcus metagenome: complete genome sequence of a macrolide-resistant serotype M6 strain. *J. Infect. Dis*. **190,** 727–738.

Basma, H., Norrby-Teglund, A., Guedez, Y., McGeer, A., Low, D.E., El-Ahmedy, O., Schwartz, B. and Kotb, M. (1999). Risk factors in the pathogenesis of invasive group A streptococcal infections: role of protective humoral immunity. *Infect. Immun*. **67,** 1871–1877.

Beres, S.B., Sylva, G.L., Barbian, K.D., Lei, B., Hoff, J.S., Mammarella, N.D., Liu, M.Y., Smoot, J.C., Porcella, S.F., Parkins, L.D., Campbell, D.S., Smith, T.M., McCormick, J.K., Leung, D.Y., Schlievert, P.M. and Musser, J.M. (2002). Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence. *Proc. Natl. Acad. Sci. USA* **99,** 10078–10083.

Bessen, D., Izzo, M., Fiorentino, T., Caringal, R., Hollingshead, S. and Beall, B. (1999). Genetic linkage of exotoxin alleles and *emm* gene markers for tissue tropism in group A Streptococci. *J. Infect. Dis*. **179,** 627–636.

Bisno, A., Brito, M. and Collins, C. (2003). Molecular basis of group A Streptococcal virulence. *Lancet Infect. Dis*. **3,** 191–200.

Bohach, G., Hauser, A. and Schlievert, P. (1988). Cloning of the gene, speB, for streptococcal pyrogenic exotoxin type B in *Escherichia coli. Infect. Immun*. **56,** 1665–1667.

Bohach, G.A., Fast, D.J., Nelson, R.D. and Schlievert, P.M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit. Rev. Microbiol*. **17,** 251–272.

Braun, M., Gerlach, D., Hartwig, U., Ozegowski, J., Romagne, F., Carrel, S., Köhler, W. and Fleischer, B. (1993). Stimulation of human T cells by streptococcal "superantigen" erythrogenic toxins (scarlet fever toxins). *J. Immunol*. **150,** 2457–2466.

Broudy, T., Pancholi, V. and Fischetti, V. (2001). Induction of lysogenic bacteriophage and phage-associated toxin from group A Streptococci during coculture with human pharyngeal cells. *Infect. Immun*. **69,** 1440–1443.

Carapetis, J., Robins-Browne, R., Martin, D., Shelby-James, T. and Hogg, G. (1995). Increasing severity of invasive group A Streptococcal disease in Australia: clinical and molecular epidemiological features and identification of a new virulent M-nontypeable clone. *Clin. Infect. Dis*. **21,** 1220–1227.

Cimaz, R. and Falcini, F. (2003). An update on Kawasaki disease. *Autoimmun. Rev*. **2,** 258–263.

Cunningham, M. (2000). Pathogenesis of group A Streptococcal infections. *Clin. Microbiol. Rev*. **13,** 470–511.

De Marzi, M., Fernandez, M., Sundberg, E., Molinero, L., Zwirner, N., Llera, A., Mariuzza, R. and Malchiodi, E. (2004). Cloning, expression, and interaction of human T cell receptors with the bacterial superantigen SSA. *Eur. J. Biochem*. **271,** 4075–4083.

Dellabona, P., Peccoud, J., Kappler, J., Marrack, P., Benoist, C. and Mathis, D. (1990). Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* **62,** 1115–1121.

Dick, G. and Dick, G. (1983). Landmark article Jan 26, 1924: The etiology of scarlet fever. By George F. Dick and Gladys Henry Dick. *JAMA* **250,** 3096.

Eriksson, B., Andersson, J., Holm, S. and Norgren, M. (1999). Invasive group A streptococcal infections: T1M1 isolates expressing pyrogenic exotoxins A and B in combination with selective lack of toxin-neutralizing antibodies are associated with increased risk of streptococcal toxic shock syndrome. *J. Infect. Dis*. **180,** 410–418.

Fast, D.J., Schlievert, P.M. and Nelson, R.D. (1989). Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. *Infect. Immun*. **57,** 291–294.

Ferretti, J.J., Mcshan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Ferretti, J., Ajdic, D. and McShan, W. (2004). Comparative genomics of streptococcal species. *Indian J. Med. Res*. **119** Suppl:1–6.

Fraser, J.D., Arcus, V., Kong, P., Baker, E. and Proft T. (2000). Superantigens—powerful modifiers of the immune system. *Mol. Med. Today* **6,** 125–132.

Gerlach, D., Knoll, H., Köhler, W. and Ozegowski, J. (1981). Isolation and characterization of erythrogenic toxins of *Streptococcus pyogenes* 3. communication: comparative studies of type A erythrogenic toxins. *Zentralbl. Bakteriol. Mikrobiol. Hyg*. [A] **250,** 277–286.

Gerlach, D., Knoll, H., Köhler, W., Ozegowski, J. and Hribalova, V. (1983). Isolation and characterization of erythrogenic toxins. V. Communication: identity of erythrogenic toxin type B and streptococcal proteinase precursor. *Zentralbl. Bakteriol. Mikrobiol. Hyg*. [A] **255,** 221–233.

Gerlach, D., Reichardt, W., Fleischer, B. and Schmidt, K. (1994). Separation of mitogenic and pyrogenic activities from so-called erythrogenic toxin type B (Streptococcal proteinase). *Zentralbl. Bakteriol*. **280,** 507–514.

Gerlach, D., Fleischer, B., Wagner, M., Schmidt, K., Vettermann, S. and Reichardt, W. (2000). Purification and biochemical characterization of a basic superantigen (SPEX/SMEZ3) from *Streptococcus pyogenes. FEMS Microbiol. Lett*. **188,** 153–163.

Gerlach, D., Schmidt, K.-H. and Fleischer, B. (2001). Basic streptococcal superantigens (SPEX/SMEZ or SPE-C) are responsible for the mitogenic activity of the so-called mitogenic factor (MF). *FEMS Immunol. Med. Microbiol*. **30,** 209–216.

Goshorn, S.C., Bohach, G.A. and Schlievert, P.M. (1988). Cloning and characterization of the gene, speC, for pyrogenic exotoxin type C from *Streptococcus pyogenes. Mol. Gen. Genet*. **12,** 66–70.

Hackett, S. and Stevens, D. (1993). Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis. *J. Infect. Dis*. **168,** 232–235.

Hauser, A., Stevens, D., Kaplan, E. and Schlievert, P. (1991). Molecular analysis of pyrogenic exotoxins from *Streptococcus pyogenes* isolates associated with toxic shock-like syndrome. *J. Clin. Microbiol*. **29,** 1562–1567.

Hartwig U.F., Gerlach D. and Fleischer B. (1994) Major histocompatibility complex class II binding site for streptococcal pyrogenic (erythrogenic) toxin A. *Med. Microbiol. Immunol*. **183,** 257–264.

Herman, A., Kappler, J.W., Marrack, P. and Pullen, A.M. (1991). Superantigens: mechanism of T cell stimulation and role in immune responses. *Annu. Rev. Immunol*. **9,** 745–772.

Hogan, R.J., Van Beek, J., Broussard, D.R., Surman, S.L. and Woodland, D.L. (2001). Identification of MHC class II-associated peptides that promote the presentation of toxic shock syndrome toxin-1 to T cells. *J. Immunol*. **166,** 6514–6522.

Hooker, S. and Follensby, E. (1934). Studies on scarlet fever. II. Different toxins produced by hemolytic streptococci of scarlatinal origin. *J. Immunol*. **27,** 177–193.

Hsueh, P., Wu, J., Tsai, P., Liu, J., Chuang, Y. and Luh, K. (1998). Invasive group A streptococcal disease in Taiwan is not associated with the presence of streptococcal pyrogenic exotoxin genes. *Clin. Infect. Dis*. **26,** 584–589.

Hudson, K.R., Tiedemann, R.E., Urban, R.G., Lowe, S.C., Strominger, J.L. and Fraser, J.D. (1995). Staphylococcal enterotoxin A has two cooperative binding sites on major histocompatibility complex class II. *J. Exp. Med*. **182,** 711–720.

Ikebe, T., Wada, A., Inagaki, Y., Sugama, K., Suzuki, R., Tanaka, D., Tamari, A., Fujinaga, Y., Abe, Y., Shimuzu, Y. and Watanabe, H. (2002). Dissemination of the phage-associated novel superantigen gene speL in recent invasive and noninvasive *Streptococcus pyogenes* M3/T3 isolates in Japan. *Infect. Immun*. **70,** 3227–3233.

Imanishi, K., Igarashi, H. and Uchiyama, T. (1990). Activation of murine T cells by streptococcal pyrogenic exotoxin type A. Requirement for MHC class II molecules on accessory cells and identification of V beta elements in T cell receptor of toxin-reactive T cells. *J. Immunol*. **145,** 3170–3176.

Jardetzky, T.S., Brown, J.H., Gorga, J.C., Stern, L.J., Urban, R.G., Chi, Y.I., Stauffacher, C., Strominger, J.L. and Wiley, D.C. (1994). Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature* **368,** 711–718.

Jupin, C., Anderson, S., Damais, C., Alouf, J.E. and Parant, M. (1988). Toxic shock syndrome toxin as an inducer of human tumor necrosis factors and γ interferon. *J. Exp. Med*. **167,** 752–761.

Kalia, A., Enright, M., Spratt, B. and Bessen, D. (2001). Directional gene movement from human-pathogenic to commensal-like streptococci. *Infect. Immun*. **69,** 4858–4869.

Kalia, A. and Bessen, D.E. (2003). Presence of streptococcal pyrogenic exotoxin A and C genes in human isolates of group G streptococci. *FEMS Microbiol. Let*. **219,** 291–295.

Kamezawa, Y., Nakahara, T., Nakano, S., Abe, Y., Nozaki-Renard, J. and Isono, T. (1997). Streptococcal mitogenic exotoxin Z, a novel acidic superantigenic toxin produced by a T1 strain of *Streptococcus pyogenes*. *Infect. Immun*. **65,** 3828–3833.

Kapur, V., Nelson, K., Schlievert, P., Selander, R. and Musser, J. (1992). Molecular population genetic evidence of horizontal spread of two alleles of the pyrogenic exotoxin C gene (speC) among pathogenic clones of *Streptococcus pyogenes*. *Infect. Immun*. **60,** 3513–3517.

Kazmi, S.U., Kansal, R., Aziz, R.K., Hooshdaran, M., Norrby-Teglund, A., Low, D.E., Halim, A.B. and Kotb, M. (2001). Reciprocal, temporal expression of SpeA and SpeB by invasive M1T1 group A streptococcal isolates *in vivo*. *Infect. Immun*. **69,** 4988–4995.

Kim, M. and Schlievert, P. (1997). Molecular genetics, structure, and immunobiology of streptococcal pyrogenic exotoxin A and C. In: *Superantigens. Molecular Biology, Immunobiology and Relevance to Human Disease* (eds. D.Y.M. Leung, B.Huber and P.M. Schlievert), pp. 257–279. Marcel Dekker, New York.

Kim, Y. and Watson, D. (1970). A purified group A streptococcal pyrogenic exotoxin. Physiochemical and biological properties, including the enhancement of susceptibility to endotoxin lethal shock. *J. Exp. Med*. **131,** 611–622.

Kline, J. and Collins, C. (1996). Analysis of the superantigenic activity of mutant and allelic forms of streptococcal pyrogenic exotoxin A. *Infect. Immun*. **64,** 861–869.

Kline, J. and Collins, C. (1997). Analysis of the interaction between the bacterial superantigen streptococcal pyrogenic exotoxin A (SpeA) and the human T-cell receptor. *Mol. Microbiol*. **24,** 191–202.

Köhler, W. (1990). Streptococcal toxic shock syndrome. *Zentralbl. Bakteriol*. **272,** 257–264.

Konishi, N., Baba, K., Abe, J., Maruko, T., Waki, K., Takeda, N. and Tanaka, M. (1996). A case of Kawasaki disease with coronary artery aneurysms documenting *Yersinia pseudotuberculosis* infection. *Acta Paediatr*. **86,** 661–664.

Kotb, M., Norrby-Teglund, A., McGeer, A., El-Sherbini, H., Dorak, M.T., Khurshid, A., Green, K., Peeples, J., Wade, J., Thomson, G., Schwartz, B. and Low, D.E. (2002). An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections. *Nature Med*. **8,** 1398–1404.

Kreiswirth, B., Handley, J., Schlievert, P. and Novick, R. (1987). Cloning and expression of streptococcal pyrogenic exotoxin A and staphylococcal toxic shock syndrome toxin-1 in *Bacillus subtilis*. *Mol. Gen. Genet*. **208,** 84–87.

Lavoie, P.M., Thibodeau, J., Erard, F. and Sekaly, R.P. (1999). Understanding the mechanism of action of bacterial superantigens from a decade of research. *Immunol. Rev*. **168,** 257–269.

Lee, P. and Schlievert, P. (1989). Quantification and toxicity of group A streptococcal pyrogenic exotoxins in an animal model of toxic shock syndrome-like illness. *J. Clin. Microbiol*. **27,** 1890–1892.

Leonard, B., Lee, P., Jenkins, M. and Schlievert, P. M. (1991). Cell and receptor requirements for streptococcal pyrogenic exotoxin T cell mitogenicity. *Infect. Immun*. **59,** 1210–1214.

Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (1998). Three-dimensional structure of the complex between a T cell receptor beta chain and the superantigen staphylococcal enterotoxin B. *Immunity* **9,** 807–816.

Li, P.L., Tiedemann, R.E., Moffat, S.L. and Fraser, J.D. (1997). The superantigen streptococcal pyrogenic exotoxin C (SPE-C) exhibits a novel mode of action. *J. Exp. Med*. **186,** 375–383.

Li, Y., Li, H., Dimasi, N., McCormick, J.K., Martin, R., Schnuck, P., Schlievert, P.M. and Mariuzza, R.A. (2001). Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity* **14,** 93–104.

Llewelyn, M. and Cohen, J. (2002). Superantigens: microbial agents that corrupt immunity. *Lancet Infect. Dis*. **2,** 156–162.

Llewelyn, M., Sriskandan, S., Peakman, M., Ambrozak, D., Douek, D., Kwok, W., Cohen, J. and Altmann, D. (2004). HLA class II polymorphisms determine responses to bacterial superantigens. *J. Immunol*. **172,** 1719–1726.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248,** 705–711.

McCormick, J., Pragman, A., Stolpa, J., Leung, D. and Schlievert, P. (2001a). Functional characterization of streptococcal pyrogenic exotoxin J, a novel superantigen. *Infect. Immun*. **69,** 1381–1388.

McCormick, J.K., Yarwood, J.M. and Schlievert, P.M. (2001b). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55,** 77–104.

McDonald, M., Currie, B. and Carapetis, J. (2004). Acute rheumatic fever: a chink in the chain that links the heart to the throat? *Lancet Infect. Dis*. **4,** 240–245.

Miethke, T., Wahl, C., Heeg, K., Echtenacher, B., Krammer, P. and Wagner, H. (1992). T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: Critical role of tumor necrosis factor. *J. Exp. Med*. **175,** 91–98.

Miyoshi-Akiyama, T., Zhao, J., Kato, H., Kikuchi, K., Totsuka, K., Kataoka, Y., Katsumi, M. and Uchiyama, T. (2003). *Streptococcus dysgalactiae*-derived mitogen (SDM), a novel bacterial superantigen: characterization of its biological activity and predicted tertiary structure. *Mol. Microbiol*. **47,** 1589–1599.

Mollick, J.A., Miller, G.G., Musser, J.M., Cook, R.G., Grossman, D. and Rich, R.R. (1993). A novel superantigen isolated from pathogenic strains of *Streptococcus pyogenes* with aminoterminal homology to staphylococcal enterotoxins B and C. *J. Clin. Invest*. **92,** 710–719.

Müller-Alouf, H., Alouf, J.E., Gerlach, D., Ozegowski, J.H., Fitting, C. and Cavaillon, J.M. (1996). Human pro- and anti-inflammatory cytokine patterns induced by *Streptococcus pyogenes* erythrogenic (pyrogenic) exotoxins A and C superantigens. *Infect. Immun*. **64,** 1450–1453.

Müller-Alouf, H., Gerlach, D., Desreumaux, P., Leportier, C., Alouf, J.E. and Capron, M. (1997). Streptococcal pyrogenic exotoxin A (SPE A) superantigen induced production of hematopoietic cytokines, IL-12 and IL-13, by human peripheral blood mononuclear cells. *Microb. Pathog*. **23,** 265–272.

Müller-Alouf, H., Proft, T., Zollner, T., Gerlach, D., Champagne, E., Desreumaux, P., Fitting, C., Geoffroy-Fauvet, C., Alouf, J. E. and Cavaillon, J. (2001). Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. *Infect. Immun*. **69,** 4141–4145.

Nakagawa, I., Kurokawa, K., Yamashita, A., Nakata, M., Tomiyasu, Y., N, O., Kawabata, S., Yamazaki, K., Shiba, T., Yasunaga, T., Hayashi, H., Hattori, M. and Hamada, S. (2003). Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. *Genome Res*. **13,** 1042–1055.

Nelson, K., Schlievert, P., Selander, R. and Musser, J. (1991). Characterization and clonal distribution of four alleles of the speA gene encoding pyrogenic exotoxin A (scarlet fever toxin) in *Streptococcus pyogenes. J. Exp. Med*. **174,** 1271–1274.

Norrby-Teglund, A., Holm, S. and Norgren, M. (1994a). Detection and nucleotide sequence analysis of the speC gene in Swedish clinical group A streptococcal isolates. *J. Clin. Microbiol*. **32,** 705–709.

Norrby-Teglund, A., Newton, D., Kotb, M., Holm, S. and Norgren, M. (1994b). Superantigenic properties of the group A streptococcal exotoxin SpeF (MF). *Infect. Immun*. **62,** 5227–5233.

Norrby-Teglund, A., Nepom, G.T. and Kotb, M. (2002). Differential presentation of group A streptococcal superantigens by HLA class II DQ and DR alleles. *Eur. J. Immunol*. **32,** 2570–2577.

Oster, H. and Bisno, A. (2000). Group C and G streptococcal infections: epidemiologic and clinical aspects. In: *Gram-Positive Pathogens* (eds. J.J. Fischetti, R. Novick, J.J. Ferretti, D. Portnoy and J. Rood), pp. 184–190. ASM Press, Washington, D.C.

Papageorgiou, A.C., Collins, C.M., Gutman, D.M., Kline, J.B., O'Brian, S.M., Tranter, H.S. and Acharya, K.R. (1999). Structural basis for the recognition of superantigen streptococcal pyrogenic exotoxin A (SpeA) by MHC class II molecules and T cell receptors. *EMBO J*. **18,** 9–21.

Petersson, K., Hakansson, M., Nilsson, H., Forsberg, G., Svensson, L.A., Liljas, A. and Walse, B. (2001). Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *EMBO J*. **20,** 3306–3312.

Podbielski, A., Woischnik, M., Leonard, B.A. and Schmidt, K.H. (1999). Characterization of nra, a global negative regulator gene in group A streptococci. *Mol. Microbiol*. **31,** 1051–1064.

Proft, T., Moffatt, S.L., Berkahn, C.J. and Fraser, J.D. (1999). Identification and characterization of novel superantigens from *Streptococcus pyogenes. J. Exp. Med*. **189,** 89–101.

Proft, T., Moffatt, S., Weller, K., Paterson, A., Martin, D. and Fraser, J. (2000). The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation. *J. Exp. Med*. **191,** 1765–1776.

Proft, T., Arcus, V., Handley, V., Baker, E. and Fraser, J. (2001). Immunological and biochemical characterization of streptococcal pyrogenic exotoxins I and J (SPE-I and SPE-J) from *Streptococcus pyogenes. J. Immun*. **166,** 6711–6719.

Proft, T., Sriskandan, S., Yang, L. and Fraser, J. (2003a). Superantigens and streptococcal toxic shock syndrome. *Emerg. Infect. Dis*. **9,** 1211–1218.

Proft, T., Webb, P.D., Handley, V. and Fraser, J.D. (2003b). Two novel superantigens found in both group A and group C *Streptococcus. Infect. Immun*. **71,** 1361–1369.

Reichardt, W., Müller-Alouf, H., Alouf, J. and Köhler, W. (1992). Erythrogenic toxins A, B, and C: occurrence of the genes and exotoxin formation from clinical *Streptococcus pyogenes* strains associated with streptococcal toxic shock-like syndrome. *FEMS Microbiol. Lett*. **79,** 313–322.

Rikiishi, H., Okamoto, S., Sugawara, S., Tamura, K., Liu, Z. and Kumagai, K. (1997). Superantigenicity of helper T cell mitogen (SPM-2) isolated from culture supernatants of *Streptococcus pyogenes. Immunology* **91,** 406–413.

Rink, L., Luhm, J., Koester, M. and Kirchner, H. (1996). Induction of a cytokine network by superantigens with parallel TH1 and TH2 stimulation. *J. Interferon Cytokine Res*. **16,** 41–47.

Roussel, A., Anderson, B.F., Baker, H.M., Fraser, J.D. and Baker, E.N. (1997). Crystal structure of the streptococcal superantigen SPE-C: dimerization and zinc binding suggest a novel mode of interaction with MHC class II molecules. *Nature Struct. Biol*. **4,** 635–643.

Sachse, S., Seidel, P., Gerlach, D., Gunther, E., Rodel, J., Straube, E. and Schmidt, K. (2002). Superantigen-like gene(s) in human pathogenic *Streptococcus dysgalactiae*, subsp equisimilis: genomic localization of the gene encoding streptococcal pyrogenic exotoxin G (speG(dys)). *FEMS Immunol. Med. Microbiol*. **34,** 159–167.

Schlievert, P. (1982). Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C. *Infect. Immun*. **36,** 123–128.

Schlievert, P., Bettin, K. and Watson, D. (1979). Reinterpretation of the Dick test: role of group A streptococcal pyrogenic exotoxin. *Infect. Immun*. **26,** 467–472.

Schlievert, P. and Gray, E. (1989). Group A streptococcal pyrogenic exotoxin (scarlet fever toxin) type A and blastogen A are the same protein. *Infect. Immun*. **57,** 1865–1867.

Schlievert, P., Assimacopoulos, A. and Cleary, P. (1996). Severe invasive group A streptococcal disease: clinical description and mechanisms of pathogenesis. *J. Lab. Clin. Med*. **127,** 13–22.

Schuh, V., Hribalova, V. and Atkins, E. (1970). The pyrogenic effect of scarlet fever toxin. IV. Pyrogenicity of strain C 203 U filtrate: comparison with some basic characteristics of the known types of scarlet fever toxin. *Yale J. Biol. Med*. **43,** 31–42.

Schwartz, B., Facklam, R. and Breiman, R. (1990). Changing epidemiology of group A streptococcal infection in the USA. *Lancet* **336,** 1167–1171.

Seal, D. (2001). Necrotizing fasciitis. *Curr. Opin. Infect. Dis*. **14,** 127–132.

Seth, A., Stern, L.J., Ottenhoff, T.H., Engel, I., Owen, M.J., Lamb, J.R., Klausner, R.D. and Wiley, D.C. (1994). Binary and ternary complexes between T cell receptor, class II MHC, and superantigen *in vitro. Nature* **369,** 324–327.

Smoot, J.C., Barbian, K.D., Van Gompel, J.J., Smoot, L.M., Chaussee, M.S., Sylva, G.L., Sturdevant, D.E., Ricklefs, S.M., Porcella, S.F., Parkins, L.D., Beres, S.B., Campbell, D.S., Smith, T.M., Zhang, Q., Kapur, V., Daly, J.A., Veasy, L.G. and Musser, J.M. (2002a). Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proc. Natl. Acad. Sci. USA* **99,** 4668–4673.

Smoot, L.M., McCormick, J.K., Smoot, J.C., Hoe, N.P., Strickland, I., Cole, R.L., Barbian, K.D., Earhart, C.A., Ohlendorf, D.H., Veasy, L.G., Hill, H.R., Leung, D.Y.M., Schlievert, P.M. and Musser, J.M. (2002b). Characterization of two novel pyrogenic toxin superantigens made by an acute rheumatic fever clone of *Streptococcus pyogenes* associated with multiple disease outbreaks. *Infect. Immun*. **70,** 7095–7104.

Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A. and McLaughlin, R. (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes. Proc. Natl. Acad. Sci. USA* **98,** 4658–4663.

Sriskandan, S., Moyes, D., Buttery, L., Krausz, T., Evans, T., Polak, J. and Cohen, J. (1996a). Streptococcal pyrogenic exotoxin A release, distribution, and role in a murine model of fasciitis and multiorgan failure due to *Streptococcus pyogenes. J. Infect. Dis*. **173,** 1399–1407.

Sriskandan, S., Moyes, D. and Cohen, J. (1996b). Detection of circulating bacterial superantigen and lymphotoxin-A in patients with streptococcal toxic shock syndrome. *Lancet* **348,** 1315–1316.

Sriskandan, S., Unnikrishnan, M., Krausz, T. and Cohen, J. (2000). Mitogenic factor (MF) is the major DNase of serotype M89 *Streptococcus pyogenes*. *Microbiology* **146,** 2785–2792.

Sriskandan, S., Unnikrishnan, M., Krausz, T., Dewchand, H., Van Noorden, S., Cohen, J. and Altmann, D. (2001). Enhanced susceptibility to superantigen-associated streptococcal sepsis in human leukocyte antigen-DQ transgenic mice. *J. Infect. Dis*. **184,** 166–173.

Stevens, D.L. (1992). Invasive group A streptococcus infections. *Clin. Infect. Dis*. **14,** 2–13.

Stevens, D.L. (1995). Streptococcal toxic shock syndrome: spectrum of disease, pathogenesis, and new concepts in treatment. *Emerg. Infect. Dis*. **1,** 69–78.

Stevens, D.L., Bryant, A., Hackett, S., Chang, A., Peer, G., Kosanke, S., Emerson, T. and Hinshaw, L. (1996). Group A streptococcal bacteremia: the role of tumor necrosis factor in shock and organ failure. *J. Infect. Dis*. **173,** 619–626.

Stevens, D.L. (2000). Streptococcal toxic shock syndrome associated with necrotizing fasciitis. *Annu. Rev. Med*. **51,** 271–288.

Stevens, K., Van, M., Lamphear, J. and Rich, R. (1996). Altered orientation of streptococcal superantigen (SSA) on HLA-DR1 allows unconventional regions to contribute to SSA Vbeta specificity. *J. Immunol*. **157,** 4970–4978.

Stock, A. and Lynn, R. (1961). Preparations and properties of partially purified erythrogenic toxin B of group A streptococci. *J. Immunol*. **86,** 561–566.

Sundberg, E.J. and Jardetzky, T. (1999). Structural basis for HLA-DQ binding by the streptococcal superantigen SSA. *Nat. Struct. Biol*. **6,** 123–129.

Sundberg, E.J., Li, H., Llera, A.S., McCormick, J.K., Tormo, J., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (2002a). Structures of two streptococcal superantigens bound to TCR β chains reveal diversity in the architecture of T cell signaling complex. *Structure* **10,** 687–699.

Sundberg, E.J., Li, Y. and Mariuzza, R.A. (2002b). So many ways of getting in the way: diversity in the molecular architecture of superantigen-dependent T cell signaling complexes. *Curr. Opin. Immunol*. **14,** 36–44.

Tiedemann, R.E. and Fraser, J.D. (1996). Cross-linking of MHC class II molecules by staphylococcal enterotoxin A is essential for antigen-presenting cell and T cell activation. *J. Immunol*. **157,** 3958–3966.

Tomai, M., Schlievert, P. and Kotb, M. (1992). Distinct T cell receptor V beta gene usage by human T lymphocytes stimulated with the streptococcal pyrogenic exotoxins and pep M5 protein. *Infect. Immun*. **60,** 701–705.

Unnikrishnan, M., Altmann, D., Proft, T., Wahid, F., Cohen, J., Fraser, J. and Sriskandan, S. (2002). The bacterial superantigen streptococcal mitogenic exotoxin Z is the major immunoactive agent of *Streptococcus pyogenes*. *J. Immunol*. **169,** 2525–2569.

Watson, D. (1960). Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. *J. Exp. Med*. **111,** 255–284.

Weeks, C.R. and Ferretti, J.J. (1986). Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from *Streptococcus pyogenes* bacteriophage T12. *Infect. Immun*. **52,** 144–150.

Welcher, B., Carra, J., DaSilva, L., Hanson, J., David, C., Aman, M. and Bavari, S. (2002). Lethal shock induced by streptococcal pyrogenic exotoxin A in mice transgenic for human leukocyte antigen-DQ8 and human CD4 receptors: implications for development of vaccines and therapeutics. *J. Infect. Dis*. **186,** 501–510.

Wen, R., Cole, G.A., Surman, S., Blackman, M.A. and Woodland, D.L. (1996). Major histocompatibility complex class II-associated peptides control the presentation of bacterial superantigens to T cells. *J. Exp. Med*. **183,** 1083–1092.

White, J., Herman, A., Pullen, A., Kubo, R., Kappler, J. and Marrack, P. (1989). The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56,** 27–35.

Willoughby, R. and Greenberg, R. (1983). The toxic shock syndrome and streptococcal pyrogenic exotoxins. *Ann. Intern. Med*. **98,** 559.

Working Group on Severe Streptococcal Infections (1993). Defining the group A streptococcal toxic shock syndrome. Rationale and consensus definition. *JAMA* **269,** 390–391.

Yamaoka, J., Nakamura, E., Takeda, Y., Imamura, S. and Minato, N. (1998). Mutational analysis of superantigen activity responsible for the induction of skin erythema by streptococcal pyrogenic exotoxin C. *Infect. Immun*. **66,** 5020–5026.

Yoshioka, T., Matsutani, T., Iwagami, S., Toyosaki-Maeda, T., Yutsudo, T., Tsuruta, Y., Suzuki, H., Uemura, S., Takeuchi, T., Koike, M. and Suzuki, R. (1999). Polyclonal expansion of TCRBV2- and TCRBV6-bearing T cells in patients with Kawasaki disease. *Immunology* **96,** 465–472.

Yu, C.E. and Ferretti, J.J. (1989). Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (speA) in clinical *Streptococcus pyogenes* strains. *Infect. Immun*. **57,** 3715–3719.

Yutsudo, T., Murai, H., Gonzalez, J., Takao, T., Shimonishi, Y., Takeda, Y., Igarashi, H. and Hinuma, Y. (1992). A new type of mitogenic factor produced by *Streptococcus pyogenes*. *FEBS Lett*. **308,** 30–34.

Zabriskie, J. (1964). The role of temperate bacteriophage in the production of erythrogenic toxin by group A streptococci. *J. Exp. Med*. **119,** 761–780.

CHAPTER

# 52

# The superantigenic toxin of *Yersinia pseudotuberculosis*

*Christophe Carnoy, Nadine Lemaitre, and Michel Simonet*

## INTRODUCTION

Superantigens are produced by a range of pathogenic bacteria and viruses and elicit powerful host immune responses via an unconventional mechanism. The superantigens are presented to T cells by direct binding to major histocompatibility (MHC) molecules present on the antigen-presenting cell (APC) surface, without MHC restriction, and specifically recognize the variable region of the β chain (Vβ) of T cell receptors (TCR) (Marrack and Kappler, 1990). As a consequence of this interaction, T cells and APCs release large amounts of inflammatory cytokines, which can cause toxic shock and tissue damage (Llewelyn and Cohen, 2002). The Gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes* are considered to be the principal sources of bacterial superantigens (Müller-Alouf *et al*., 2001; Alouf *et al*., 2005). However, in 1993, a mitogen with superantigenic features was characterized in the Gram-negative bacillus *Yersinia pseudotuberculosis*.

## *YERSINIA PSEUDOTUBERCULOSIS*, AN ENTEROPATHOGENIC BACTERIUM

Malassez and Vignal first described *Y. pseudotuberculosis* in 1883 as the causative agent of tuberculosis-like lesions (pseudotuberculosis) in guinea pigs. Seventy years later, Knapp and Mashoff isolated the germ from the enlarged, ileocaecal lymph nodes of two German children who had undergone surgery for suspected appendicitis. There have been many subsequent reports of human *Y. pseudotuberculosis* infections in Europe, North America, Japan, and Eastern Russia. With the exception of a few outbreaks in Finland, Eastern Russia, and Japan (Somov and Martinevsky, 1973; Inoue *et al*., 1984; Tertti *et al*., 1984; Nakano *et al*., 1989; Pebody *et al*., 1997; Jalava *et al*., 2004; Nuorti *et al*., 2004), most cases are sporadic. In Eurasia and North America, *Y. pseudotuberculosis* is enzootic in various mammal and bird species, which may harbor this microorganism as healthy carriers (Fukushima and Gomyoda, 1991). Human infection is most probably established by ingestion of food or water contaminated by the excreta of infected animals. In immunocompetent hosts, infection is self-limited and causes relatively mild illness. The most frequent clinical symptoms are fever and abdominal pain rather than the diarrhea and vomiting usually associated with gastrointestinal infections. Abdominal pain in the right fossa mimics acute appendicitis (pseudoappendicular syndrome) and can prompt surgery; while considerable mesenteric adenitis and/or acute terminal ileitis are observed peroperatively, the appendix itself appears normal. Several cases of Crohn's ileitis following *Y. pseudotuberculosis* infection have been also reported in the literature, but whether this bacterium triggers this inflammatory bowel disease is subject to debate (Blaser *et al*., 1984; Treacher and Jewell, 1985; Homewood *et al*., 2003; Hugot *et al*., 2003). In debilitated patients (especially those with cirrhosis due to alcoholism or hemochromatosis or other liver diseases), microorganisms have the propensity to spread from the digestive

tract to deep organs via the blood, and septicemia is often fatal (Butler, 1983).

A typical feature of *Y. pseudotuberculosis* infection is the occurrence of post-infection complications, such as reactive arthritis and erythema nodosum (Butler, 1983; Tertti *et al*., 1984; Tertti *et al*., 1989). Commonly, arthritis lasts for six months, and the clinical course of the illness can range from an acute, self-limiting form in most cases to intermittent, relapsing, or chronic arthritis in a few cases (Simonet, 1999). Erythema nodosum, sometimes associated with reactive arthritis, is characterized by typical aseptic and inflammatory nodules on the patient's legs. In Japan, where pseudotuberculosis is often epidemic, infection in children frequently associates fever, conjunctival injection, a biphasic erythematous rash, lip changes (fissure, redness, or crust), induration of hands or feet, erythema of palms of the hands or the soles of the feet, desquamation of the peripheral extremities, strawberry tongue, inflamed oral mucosa, enlarged lymph nodes, erythema nodosum, and arthritis (Sato *et al*., 1983). These clinical manifestations, rarely reported in European countries, resemble Kawasaki disease and the Far East scarlet fever disease reported in Eastern Russia during the 1960s (Somov and Martinevsky, 1973). It has been suggested that *Y. pseudotuberculosis* might be a possible but non-exclusive etiologic agent of these illnesses. Finally, approximately 10% of *Y. pseudotuberculosis*-infected patients develop renal complications. The most frequent one (80% of cases) is acute renal failure, probably due to tubulointerstitial nephritis, but acute nephritic syndrome, IgA nephropathy, and hemolytic uraemic syndrome have been also described (Takeda *et al*., 1991; Cheong *et al*., 1995).

## *Y. PSEUDOTUBERCULOSIS* PRODUCES AN EXOTOXIN WITH SUPERANTIGEN ACTIVITY

The typical post-infection immunopathological complications that may occur after infection with *Y. pseudotuberculosis* (reactive arthritis, erythema nodosum, Kawasaki disease, etc.) suggested the involvement of a superantigen-like molecule. In 1993, a substance with mitogenic activity on human peripheral blood mononuclear cells (PBMCs) was simultaneously described in a *Y. pseudotuberculosis* strain involved in a mass outbreak in Japan (Uchiyama *et al*., 1993) and in an isolate from a patient presenting Kawasaki-like symptoms (Abe *et al*., 1993; Yoshino *et al*., 1994). The substance was designated YPM for "*Y. pseudotuberculosis*-derived mitogen" (Miyoshi-Akiyama *et al*., 1993). YPM has a molecular weight of 14,524 as demonstrated by electrospray ionization mass spectrometry (Yoshino *et al*., 1994). Cloning and sequencing of the corresponding gene revealed a 456-base pair (bp) open reading frame (*ypm*) that codes for a 151 amino acid product, which is then processed into a mature 131-residue form after cleavage of a 20 amino acid hydrophobic signal sequence (Figure 52.1) (Ito *et al*., 1995; Miyoshi-Akiyama *et al*., 1995). The presence of a signal peptide suggests that YPM is secreted via the general secretory pathway found in most Gram-negative bacteria.

YPM expands human T lymphocytes expressing the Vβ3, Vβ9, Vβ13.1, and Vβ13.2 variable regions (Abe *et al*., 1993; Uchiyama *et al*., 1993) with a preference for the $CD4^+$ T cells, although some $CD8^+$ lymphocytes can also proliferate (Ito *et al*., 1995). YPM is active at concentrations as low as 1pg/ml (Ito *et al*., 1995), and requires the presence of MHC class II molecules to be mitogenic. This is evidenced by the observation that (i) YPM stimulates T cells in the presence of fibroblasts transfected with HLA-DP, HLA-DQ and HLA-DR class II molecules, with a higher degree of expansion in the presence of HLA-DR (DR4 subtype) and (ii) YPM-induced T lymphocyte expansion can be inhibited by monoclonal antibodies directed against HLA-DR (Abe *et al*., 1993; Uchiyama *et al*., 1993). Paraformaldehyde fixation of HLA-DR-transfected fibroblasts does not alter YPM-induced cellular stimulation, suggesting that YPM binds directly to HLA class II molecules without being processed (Abe *et al*., 1993; Uchiyama *et al*., 1993). Taken as a whole, these studies suggest that the mitogen produced by *Y. pseudotuberculosis* displays all the criteria of a superantigenic toxin as defined by Marrack and Kappler (1990).

```
YPMa    MKNKLLSLLTFTLFSGVALATDYDNTLNSIPSLRIPNIATYTGTIQGKGEVCIIGNKEGK  60
YPMc    MKNKLLSLLTFTLFSGVALATDYDNTLNSIPSLRIPNIATYTGTIQGKGEVCIIGNKEGK  60
YPMb    MKKKFLSLLTLTFFSGLALAADYDNTLNSIPSLRIPNIETYTGTIQGKGEVCIRGNKEGK  60

YPMa    TRGGELYAVLHSTNVNADMTLILLRNVGGNGWGEIKRNDIDKPLKYEDYYTSG-LSWIWK 119
YPMc    TRGGELYAVLYSTNVNADMTLILLRNVGGNGWGEIKRNDIDKPLKYEDYYTSG-LSWIWK 119
YPMb    SRGGELYAVLRSTNANADMTLILLCSIR-DGWKEVKRSDIDRPLRYEDYYTPGALSWIWE 119

YPMa    IKNNSSETSNYSLDATVHDDKEDSDVLTKCPV 151
YPMc    IKNNSSETSNYSLDATVHDDKEDSDVLTKCPV 151
YPMb    IKNNSSEASDYSLSATVHDDKEDSDVLMKCP- 150
```

**FIGURE 52.1** YPM variant amino acid sequences deduced from the nucleotide sequence of *ypmA, ypmB,* and *ypmC*. Amino acids are represented by a single letter code. Shaded letters correspond to the amino acid residues that differ from the YPMa reference sequence. YPMa signal sequence is underlined.

## THE THREE YPM VARIANTS ARE UNRELATED TO OTHER BACTERIAL SUPERANTIGENS

A few years after the initial characterization of YPM, Ramamurthy *et al*. (1997) reported that some mitogenic *Y. pseudotuberculosis* isolates failed to hybridize with a *ypm* probe and showed that these strains harbored a gene coding for a 150 amino acid protein sharing 83% homology with YPM. As a result of this report, the first described *Y. pseudotuberculosis* superantigen was called YPMa and the second variant was referred to as YPMb. The lowest amino acid homology between these YPM variants is seen in the central part of the mature protein, between amino acids 65 and 86 (Figure 52.1). YPMa contains two cysteines (at positions 32 and 129), whereas YPMb possesses an additional cysteine residue at position 66. These differences do not impair TCR recognition since YPMa and YPMb both stimulate T cells bearing Vβ 3, 9, 13.1, and 13.2 regions (Ramamurthy *et al*., 1997). Screening a *Y. pseudotuberculosis* strain collection by hybridization under mild stringency conditions with a *ypmA*-specific probe, followed by sequencing of the internal region of the superantigen encoding genes, enabled the authors to discover another *ypm* allele with a single nucleotide difference relative to *ypmA*. This new variant, in which the histidine residue at position 51 of the mature protein is replaced by tyrosine, was designated YPMc (Figure 52.1) (Carnoy and Simonet, 1999; Carnoy *et al*., 2002). *ypmA* is by far the most frequent allele, since it is found in 83% of superantigenic isolates, whereas *ypmB* and *ypmC* are respectively present in 5% and 12% of these strains (Fukushima *et al*., 2001). Molecular typing of the YPM producers, based on analysis of the genomic distribution of the IS*1541* insertion sequence, strongly suggested that the *ypmB* allele is the ancestral gene and that *ypmC* recently emerged from a *ypmA*-expressing clone (Carnoy and Simonet, 1999). This latter finding is consistent with the exclusive presence of *ypmC* in serotype O:3 strains (Fukushima *et al*., 2001).

The small size of the YPM toxins (14.5 kDa) compared to those of other bacterial superantigens, which range from 20 to 30 kDa, soon prompted the suggestion that YPMs were probably unrelated to other superantigens (Petersson *et al*., 2004). This hypothesis was confirmed by phylogenetic analysis based on protein identity of 22 superantigens that clearly demonstrated that YPMs diverged from Gram-positive superantigens and from the MAM superantigen produced by *Mycoplasma arthritidis*, a pathogen involved in arthritis in mice (Müller-Alouf *et al*., 2001).

## YPMA DISPLAYS A JELLY-ROLL FOLD

YPMa has recently been crystallized, and x-ray crystal analysis revealed a jelly-roll fold comprising two β sheets, each with four antiparallel strands (Figure 52.2) (Donadini *et al*., 2004). Nuclear magnetic resonance (NMR) analysis of YPMa in solution confirmed the three-dimensional (3D) structure. Surprisingly, the superantigen was found to be monomeric in solution, whereas it packed as a trimer in crystals (Figure 52.2). The *in vivo* relevance of the YPM trimer seen in crystals is questionable. However, YPMa trimerization might occur in other high-density molecular environments, for example when the superantigen cross-

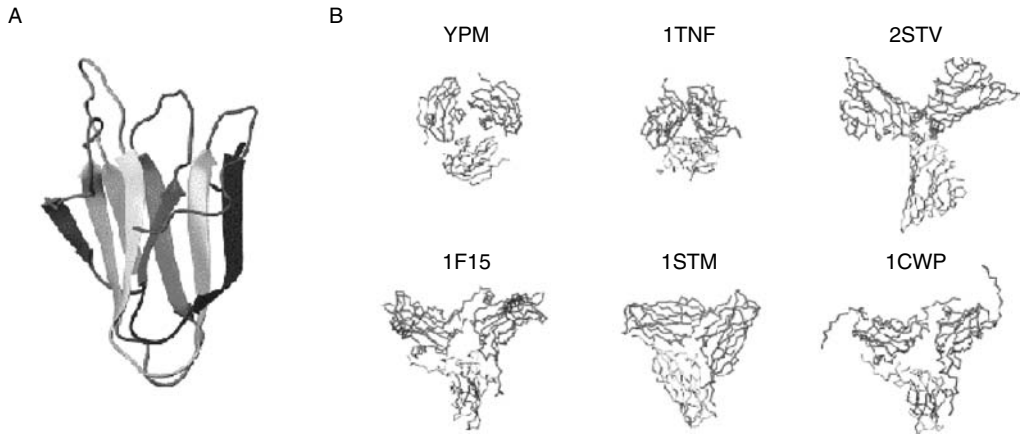



**FIGURE 52.2** Three-dimensional structure of YPMa. A. Ribbon diagram showing the secondary structure of YPMa as determined by NMR spectroscopy. B. YPMa trimer assembly in the crystal structure, compared to TNF-α (1TNF) and proteins from satellite tobacco mosaic virus (2STV), cucumber mosaic virus (1F15), satellite panicum mosaic virus (1STM), and cowpea chlorotic mottle virus (1CWP) (Donadini *et al*., 2004).

links to MHC molecules and TCRs (Donadini *et al*., 2004). Interestingly, searches for structurally related proteins indicated that YPMa exhibits strong similarities, especially when in its trimeric form, with Tumor Necrosis Factor (TNF) superfamily proteins, as well as with human, plant, and insect viral capsid proteins (Figure 52.2) (Donadini *et al*., 2004). These findings, which primary amino acid sequence homology analysis had failed to reveal, favor the hypothesis whereby YPM has a viral origin. The absence of 3D structural homology with the staphylococcal and streptococcal superantigens that have been crystallized to date (Petersson *et al*., 2004) also confirms that YPM is unique in the superantigen world.

Elucidation of YPMa 3D structure represents an important milestone in the understanding of the biological activity of this original superantigen. However, the molecular mechanisms underlying the interaction between YPM and its ligands (i.e., TCRs and MHC class II molecules) are still poorly understood, despite several structure-function studies. Initial comparison of YPMa and YPMb variants revealed a markedly different amino acid composition in the central region (residues 65 to 86) of the mature proteins (Ramamurthy *et al*., 1997) (Figure 52.1). Since both variants exhibit the same Vβ specificities, the central region was thought not to be crucial for TCR recognition. In a preliminary investigation, Yoshino *et al*. (1996) tested the blocking effect of synthetic YPMa internal peptides on superantigen-induced stimulation of PBMCs and observed that the peptide corresponding to the first 23 amino acids of the mature protein was found to be the best inhibitor. However, only 50% of YPM-triggered cellular expansion was abrogated by this peptide. This indicates that domains other than the N-terminus might also contribute to YPM cell proliferation properties. Involvement of the N-terminal region was also suggested by a deletion mutant study: Removal of 11, 19, or 29 residues at the N-terminal end of the protein abolishes the proliferative properties and affects YPM binding to HLA-DR (Ito *et al*., 1999). Further studies, based on random mutagenesis, mapped the protein residues responsible for the superantigen biological activity (Ito *et al*., 1999; Seprenyi *et al*., 1999). The most exhaustive work characterized single and double YPM mutants with low mitogenicity (less than 25% of wild-type YPM activity) (Ito *et al*., 1999). Some of them (K37N, D124G, L46S, L64R/T115I, G71E/L94I, and H117D) still bound to HLA-DR molecules, whereas other point mutants (L7Q/D79G, S52F, L112S) lost this ability. Random mutagenesis also showed that cysteine residues at positions 32 and 129 of the mature polypeptide were essential for YPMa biological activity (Ito *et al*., 1999). Of the 14 amino acid substitutions that confer low activity onto YPM, only six (E30K, K37N, Y47H, N56S, V116G, and H117D) were found to be solvent-exposed on the 3D structure of the molecule (Donadini *et al*., 2004). These residues represent good candidates for binding to MHC class II or TCR molecules. Nevertheless, there currently are not enough structure-function data to specify YPM MHC class II and TCR binding sites.

## YPM IS CHROMOSOMALLY ENCODED

Not all *Y. pseudotuberculosis* strains produce YPM (Chain *et al*., 2004). This ability, which is O-serotype independent, differs according to the isolate's geographical origin: *ypm* is detected in almost all strains (more than 95%) from Far Eastern countries and, strikingly, in only one fifth (less than 20%) of strains isolated in Western countries (Yoshino *et al*., 1995; Fukushima *et al*., 2001). This finding argues for an exogenous origin of the *Y. pseudotuberculosis ypm* gene. Many bacterial superantigen-encoding genes are either borne on plasmids, phages, or pathogenicity islands. In *Y. pseudotuberculosis* superantigenic strains, *ypm* alleles are located on the chromosome, upstream of an open reading frame with similarities to *Salmonella pagO*, a PhoP/PhoQ-activated gene (Figure 52.3) (Carnoy *et al*., 2002). A new *Y. pseudotuberculosis*-specific insertion sequence, designated IS*1398*, is present in the vicinity of the *ypmA* and *ypmC* alleles (downstream of *pagO*), but is absent in the *ypmB* allele genetic locus. No other mobile genetic elements, such as phage genes or remnants, were found in the *ypm* alleles' genetic environment. The *ypm* genes are located about 250 bp downstream of a 26-bp motif called *yrs* (for *Yersinia* recombination site), which is homologous to *dif*, a site-specific recombination target in *Escherichia coli* and a filamentous phage integration locus in *Xanthomonas campestris*. Interestingly, *yrs* contains a 19-bp consensus sequence common to many Gram-negative species, thus indicating the ubiquity of this element (Figure 52.3) (Carnoy *et al*., 2002).

Instability is an important feature of the *ypm* locus. *In vitro* experiments showed that deletions within the *ypmA* chromosomal region occur about 250 times more frequently than deletions within a housekeeping gene locus (the urease operon) (Carnoy *et al*., 2002). Characterization of six independent mutants indicated that DNA deletions (i) were distinct for each mutant and were not site-specific, indicating a random event, and (ii) always spared the *yrs* site, arguing in favor of the involvement of *yrs* in the process. Analysis of the superantigen locus in *ypm*-negative *Y. pseudotuberculosis* revealed that DNA rearrangements occurred in very few strains, suggesting that the random deletion process might be less frequent in nature than in the

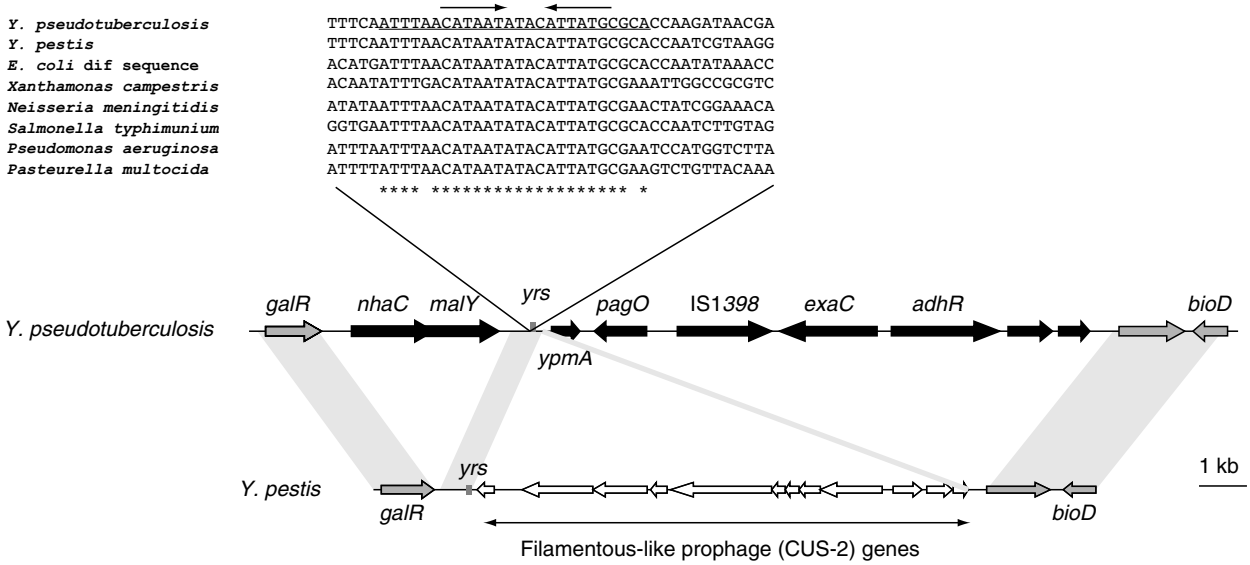



**FIGURE 52.3** Genetic organization of the *ypmA* locus from *Y. pseudotuberculosis* compared to the corresponding locus in *Y. pestis*. Gray arrows and shaded areas represent the coding sequences (CDSs) and the region common to *Y. pseudotuberculosis* and *Y. pestis*. Black-and-white arrows indicate CDSs that are specific to *Y. pseudotuberculosis* and *Y. pestis,* respectively. CDSs are identified with the name of the best hit using Blastn (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence of the *Yersinia* recombination site (*yrs*) is presented and aligned with the homologous sequences found in various Gram-negative microorganisms. Asterisks specify identical nucleotides corresponding to the consensus sequence. Arrows above the *Y. pseudotuberculosis yrs* sequence represent the two inverted repeats found in the consensus sequence (Carnoy *et al*., 2002).

laboratory. In fact, most of the non-superantigenic *Y. pseudotuberculosis* strains never acquired the *ypm* gene (Carnoy *et al*., 2002).

## *Y. PSEUDOTUBERCULOSIS* IS THE ONLY PATHOGENIC YERSINIAE THAT PRODUCES SUPERANTIGENS

In addition to *Y. pseudotuberculosis*, three other *Yersinia* species are pathogens in animals and humans: *Y. pestis*, the causative agent of plague, and *Y. enterocolitica*, another enteropathogenic microorganism. Although DNA-DNA hybridization indicates greater than 90% relatedness between *Y. pseudotuberculosis* and *Y. pestis*, Miyoshi-Akiyama *et al*. (1997) failed to detect the *ypm* gene in the latter. *In silico* analysis of three *Y. pestis* genomes further confirmed the absence of *ypm* in this species (Parkhill *et al*., 2001; Deng *et al*., 2002; Song *et al*., 2004). Interestingly, the *yrs* site is present on the chromosome of *Y. pestis*, but unlike the situation in *Y. pseudotuberculosis*, it is surrounded by filamentous phage-like (CUS-2) genes, confirming the phage integration function of the *yrs* site (Figure 52.3) (Carnoy *et al*., 2002; Gonzalez *et al*., 2002).

In the early nineties, Stuart and Woodward (1992) isolated a mitogen from a *Y. enterocolitica* lysate that stimulated mouse T cells bearing Vβ 3, 6, and 11 (and possibly 7 and 9). This protease-sensitive substance was found also to be active on human Vβ 3, 12, 14, and 17 T cells in a MHC class II-dependent manner (Stuart *et al*., 1995). Even though the *Y. enterocolitica* mitogen has been partially purified, a DNA sequence is still unavailable at present. The question of whether the mitogen shares homology with YPM remains unanswered. Nevertheless, several features argue in favor of two, distinct yersiniae superantigens. First, the species' respective mitogenicity-associated Vβ specificities are different. Second, experimental approaches have demonstrated the absence of *ypm* in more than 200 *Y. enterocolitica* strains and notably in the strain described as a mitogen producer (Yoshino *et al*., 1995; Carnoy and Simonet, 1999). Finally, *in silico* analysis of the *Y. enterocolitica* 8081 genome has not revealed any *ypm* homologues (http://www.sanger.ac.uk/Projects/Y_enterocolitica/).

## A SCENARIO FOR THE ACQUISITION OF YPM BY *Y. PSEUDOTUBERCULOSIS*

On the basis of genetic and experimental findings, an evolutionary scenario explaining the acquisition of *ypm* by *Y. pseudotuberculosis* can be proposed (Figure 52.4). Molecular typing of *Y. pseudotuberculosis* strains suggested that *ypmB* was the ancestral gene and that *ypmC* recently derived from a *ypmA* clone following a point mutation (Carnoy and Simonet, 1999). Interestingly,

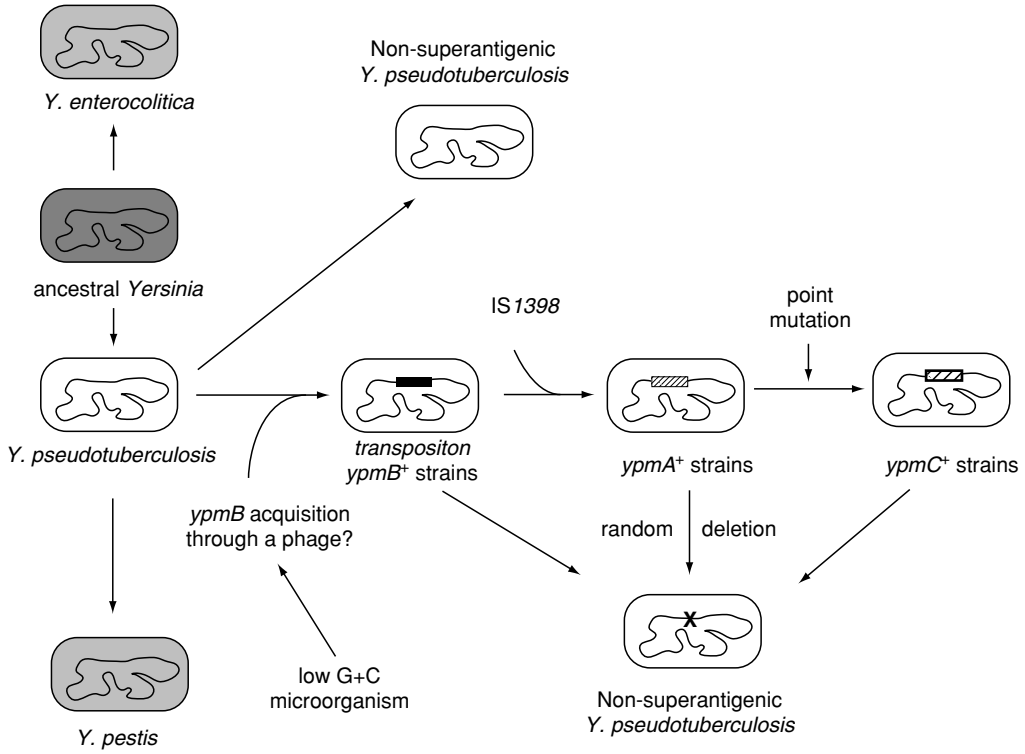



**FIGURE 52.4** A speculative scenario for *ypm* acquisition by *Y. pseudotuberculosis*. The proposed evolutionary model is based on several experimental findings: (i) *ypmB* is the ancestral gene and *ypmC*-positive strains correspond to a clonal expansion from *ypmA*-positive *Y. pseudotuberculosis*, (ii) the G+C content of the *ypmB* allele is low compared to that of the *Y. pseudotuberculosis* genome (35% versus 47%, respectively), (iii) the YPMa structure is homologous to viral capsid proteins, (iv) *ypm* alleles are located downstream of *yrs*, a phage integration site, (v) IS*1398* is never found in *ypmB*-positive strains and (vi) non-superantigenic strains can be obtained by random deletion of the *ypm* locus (Carnoy and Simonet, 1999; Carnoy *et al.*, 2002; Donadini *et al.*, 2004).

*ypmB* has only been found in strains isolated from the environment (e.g., river water) and wild animals (e.g., moles and wild mice), but never from humans (Fukushima *et al.*, 2001). This suggests that the superantigen gene ancestor would first have been acquired by non-human strains. In contrast, *ypmA* is the allele found most frequently in human isolates.

It is known that *Y. enterocolitica* and *Y. pseudotuberculosis* derive from a common ancestor and that *Y. pestis* recently emerged from *Y. pseudotuberculosis* (Achtman *et al.*, 1999). The absence of *ypm* in *Y. enterocolitica*, *Y. pestis*, and in some *Y. pseudotuberculosis* strains strongly suggests that the superantigen-encoding gene was introduced into *Y. pseudotuberculosis* after its divergence from *Y. enterocolitica*, and that *Y. pestis* derived from a non-superantigenic *Y. pseudotuberculosis* clone. This indicates that *ypm*-positive strains represent a clonal population that gained the superantigen through a horizontal gene transfer. Hence, specification of the original source of the *ypm* ancestral gene is an important issue. *ypm* genes have a guanine and cytosine (G+C) content of about 35%, whereas that of the *Y. pseudotuberculosis* genome is 47% (Carnoy *et al.*, 2002). This indicates that *ypm* might have originated from a low G+C content microorganism. Since they display low G+C contents, superantigen-producing species, such as *S. aureus*, *S. pyogenes*, and *M. arthritidis*, could be good candidates as gene donors; however, *in silico* analysis of their genomes failed to detect any putative *ypm* ancestors.

The involvement of a phage in the horizontal acquisition of *ypm* by *Y. pseudotuberculosis* is an attractive hypothesis. It is strongly supported by the location of the superantigen genes close to a phage integration site and by the presence of phage genes in the corresponding *Y. pestis* locus. Furthermore, the structural homology of the trimeric form of YPM with viral capsid proteins reinforces the idea that YPM was initially a phage-related protein. This assumption was further strengthened by a recent epidemiological study on a large *Y. pseudotuberculosis* strain collection, which revealed that *ypm* genes are closely associated with the YAPI pathogenicity island-borne *pil* operon, encoding type IV pili (Collyn *et al.*, 2005). It is known that besides their contribution to bacterial adherence, pili may serve as bacteriophage receptors (Bradley, 1973; Mindich *et al.*, 1999; Budzik *et al.*, 2004). By analogy

with *Vibrio cholerae*, in which TCP type IV pili allow attachment for enterotoxins encoding filamentous prophage CTXΦ and further bacterial lysogeny (Waldor and Mekalanos, 1996), we can speculate that *Y. pseudotuberculosis* type IV pili would be a target for *ypm*-containing phage. In this hypothesis, *Y. pseudotuberculosis* would have acquired the *pil* operon before the *ypmB* gene. Many questions remain unanswered in this model, and experimental evidence is now required to confirm the proposed evolutionary scenario.

## YPM IS A BACTERIAL VIRULENCE FACTOR

Since the discovery of YPM in 1993, very few experimental data on its role in human pathological disorders are available. A serological study established that 61% of sera from Japanese patients with acute *Y. pseudotuberculosis* infections contained elevated levels of anti-YPM antibodies (with a higher titer in patients suffering from systemic complications), demonstrating the expression of YPMa *in vivo* (Abe *et al*., 1997). In the acute phase of yersiniosis, there are higher levels of Vβ3-expressing T cells (but not those expressing Vβ9, Vβ13.1, and Vβ13.2) in the patient's blood. To explain these *in vivo* and *in vitro* discrepancies, Abe *et al*. (1997) hypothesized that of the Vβ T cells expanding in lymph nodes, only the major subset (Vβ3) can be detected in the blood. This assertion was partially confirmed by analysis of the T cells from a *Y. pseudotuberculosis*-infected patient who showed an enhancement of Vβ3 and Vβ13.2 subsets in the mesenteric lymph nodes, whereas only the Vβ3 subset was detected in the PBMCs.

A geographical correlation between the clinical mani festations and the prevalence of superantigen genes tends to point to YPM as a virulence factor. Indeed, in Europe where the frequency of isolation of superantigenic strains is low, i.e., less than 20%, *Y. pseudotuberculosis* mainly induces gastrointestinal symptoms, whereas in the Far East where the prevalence of superantigen-positive strains is high, i.e., greater than 95%, the clinical forms of illnesses are frequently more severe, with a variety of systemic manifestations, such as Izumi fever or Kawasaki disease (Fukushima *et al*., 2001).

Experimental models have been developed to assess the impact of YPM during infection with *Y. pseudotuberculosis*. Parenteral administration of purified recombinant YPM into D-galactosamine pre-sensitized C57BL/6 or BALB/c mice can induce a T cell-dependent lethal shock, as demonstrated by survival of T cell-deficient SCID mice injected with the superantigen (Miyoshi-Akiyama *et al*., 1997; Kano *et al*., 2004). The MHC class II-dependent T lymphocyte activation produces T cell blasts expressing Vβ7 or Vβ8 variable regions on the TCR (Miyoshi-Akiyama *et al*., 1997). These Vβ7, Vβ8 cells are required for the YPM-induced shock, since treatment with anti-Vβ7 or anti-Vβ8 monoclonal antibodies protects the mice from YPM toxicity. However, tissular proliferation of the two T cell subsets differs: In a murine model of long-term (7-day) exposure to YPM, it was found that $V\beta7^+$ $CD4^+$ T cells expanded at a much higher rate than $V\beta8^+$ $CD4^+$ T cells in the spleen. This indicated that YPM preferentially activated T cells carrying the Vβ7 region and had a greater affinity for the Vβ7 domain than for the Vβ8 domain. When monitoring the distribution of $V\beta8^+$ T lymphocytes in BALB/c mouse tissues, Kano *et al*. (2004) reported that the cells left the bloodstream within one hour after injection of the toxin and migrated into the liver, but not the spleen or the lymph nodes, demonstrating hepatotropism of the YPM-activated $V\beta8^+$ T cells. YPM also altered cell viability, and apoptosis occurred in the liver 12 hours after YPM administration (Kano *et al*., 2004). Nevertheless, the exact role of cellular migration and hepatocyte apoptosis in mouse lethality is still unclear and deserves further investigation. Additionally, YPM injection into BALB/c or C57BL/6 mice elicits synthesis of interferon-gamma (INF-γ) and TNF-α but not of interleukin (IL)-10. Blocking of inflammatory cytokines by anti-TNF-α and anti-IFN-γ monoclonal antibodies neutralizes YPM toxicity *in vivo*, demonstrating the involvement of these cytokines in shock (Miyoshi-Akiyama *et al*., 1997; Kano *et al*., 2004). However, overproduction of IFN-γ and TNF-α is not YPM-specific, since it is frequently associated with superantigen-mediated host immune responses (Muller-Alouf *et al*., 2001; Petersson *et al*., 2004). YPM also affects functionality of cultured colonic epithelial cells (the human T84 cell line) by increasing cell permeability and by reducing active ion transport in response to prosecretory stimuli. The biological effect, which is PBMC-dependent, can be abolished by addition of monoclonal antibodies against TNF-α and IFN-γ (Donnelly *et al*., 1999). In contrast, intrarectal instillation of YPM into BALB/c mice does not impair ion transport in the colonic mucosa, but does trigger local inflammation (Lu *et al*., 2003). Like other superantigens, YPM could be an inductor of inflammatory bowel diseases.

YPM effects may be different when mice are administered whole bacteria instead of the purified toxin. To assess the impact of YPM in the presence of *Y. pseudotuberculosis*, parenteral inoculation in OF1 mice of isogenic YPM-producing or YPM-deficient *Y. pseudotuberculosis* were performed. This experimental model revealed that the superantigen exacerbates bacterial virulence (Carnoy *et al*., 2000). In infected mice, the major immunological findings, that have been related

to bacterial YPM production are splenomegaly and IL-2 and IL-4 overproduction. Flow cytometry analysis of splenocytes from *Y. pseudotuberculosis*-infected mice indicated that YPM-dependent splenomegaly is related to T cell expansion, especially of $V\beta7^+$ and $V\beta8^+$ subsets, thus confirming the phenotype of the T cells activated *in vitro* by YPM (Carnoy *et al*., 2003). Furthermore, *in vitro* stimulation of murine thymic T cells by YPM induces blasts, which selectively express Vβ7 and Vα14 chains on the TCR (i.e., a NKT cell phenotype) and which produce substantial amounts of IL-2 and IL-4 (Yagi *et al*., 1999). Therefore, it is tempting to speculate that NKT cells might support YPM-induced toxicity in the mouse, but, to date, experimental evidence in support of this attractive hypothesis is lacking. In *Y. pseudotuberculosis*-infected mice, YPM induces neither IFN-γ overproduction nor TNF-α synthesis in the spleen and blood. This contrasts with the cytokine expression profile of animals injected with the toxin alone. Abrogation of TNF-α production in the animals after injection of *Y. pseudotuberculosis* can, in fact, be attributed to YopJ, a toxin which inhibits NF-κB activation and consequently suppresses TNF-α production (Palmer *et al*., 1998; Schesser *et al*., 1998).

## DOES YPM TRIGGER KAWASAKI DISEASE?

First described in the 1960s, Kawasaki disease is an acute multisystem vasculitis complicated by the development of coronary abnormalities (including diffuse dilatation and aneurysm formation), which primarily affects children under five years of age (Kawasaki, 1967; Kawasaki *et al*., 1974). The disease's clinical symptoms and epidemiology (with the predominantly seasonal occurrence of epidemics) strongly suggest that an infectious agent is the cause, or at least an inciting agent (Burns and Globé, 2004). Kawasaki disease is associated with elevated levels of various inflammatory cytokines and the selective expansion of some specific, Vβ-expressing T lymphocytes in the patient's peripheral blood during the active phase of the illness. The condition is also associated with the development of auto-antibodies, especially anti-endothelial cell antibodies. Taken as a whole, these features are characteristic of superantigen-induced disease. *Y. pseudotuberculosis* was cited as a possible etiologic agent, since the germ was recovered from patients with clinical manifestations resembling those of Kawasaki disease (Sato *et al*., 1983; Baba *et al*., 1991; Konishi *et al*., 1997). Additionally, YPMa was actually purified from a strain of *Y. pseudotuberculosis* isolated from a patient suffering from this illness (Yoshino *et al*., 1994). However, neither an increase or decrease in the T cell subsets bearing the Vβs recognized by YPMs (Vβ 3, 9, 13.1, and 13.2) nor the presence of anti-YPM antibodies has been described in patients suffering from Kawasaki disease.

## CONCLUSION

The discovery of a superantigen produced by *Y. pseudotuberculosis* brought new insights to the understanding of the post-infection, immunopathological complications frequently associated with this Gram-negative microorganism. Although structurally and genetically unrelated to streptococcal, staphylococcal, and mycoplasmal superantigens, YPM exhibits all the classical features of a superantigenic toxin (absence of APC processing, requirement for MHC class II molecules, Vβ-specific T cell stimulation). The YPM structural and genetic originality raises the central question of what makes a molecule a superantigen. YPM interaction with its ligands is still unknown but deserves serious attention. The recent characterization of the toxin 3D structure will undoubtedly unveil the molecular mechanism responsible for YPM-mediated mitogenicity. Furthermore, although the toxicity of YPM has been extensively demonstrated in experimental models, the role of this superantigen in *Y. pseudotuberculosis*-associated immunopathologies, especially Kawasaki disease, remains to be proven.

## REFERENCES


Abe, J., Takeda, T., Watanabe, Y., Nakao, H., Kobayashi, N., Leung, D.Y. and Kohsaka, T. (1993). Evidence for superantigen production by *Yersinia pseudotuberculosis*. *J. Immunol*. **151,** 4183–4188.

Abe, J., Onimaru, M., Matsumoto, S., Noma, S., Baba, K., Ito, Y., Kohsaka, T. and Takeda, T. (1997). Clinical role for a superantigen in *Yersinia pseudotuberculosis* infection. *J. Clin. Invest*. **99,** 1823–1830.

Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A. and Carniel, E. (1999). *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA* **96,** 14043–14048.

Alouf, J. E., and Muller-Alouf, H., (2005). What are superantigens? In *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 3rd. Alouf, J. E. and Popoff, M. R. (eds). Elsevier Inc.

Baba, K., Takeda, N. and Tanaka, M. (1991). Cases of *Yersinia pseudotuberculosis* infection having diagnostic criteria of Kawasaki disease. *Contrib. Microbiol. Immunol*. **12,** 292–296.

Blaser, M.J., Miller, R.A., Lacher, J. and Singleton, J.W. (1984). Patients with active Crohn's disease have elevated serum antibodies to antigens of seven enteric bacterial pathogens. *Gastroenterology* **87,** 888–894.

Bradley, D.E. (1973). A pilus-dependent *Pseudomonas aeruginosa* bacteriophage with a long non-contractile tail. *Virology* **51,** 489–492.

Budzik, J.M., Rosche, W.A., Rietsch, A. and O'Toole, G.A. (2004). Isolation and characterization of a generalized transducing

phage for *Pseudomonas aeruginosa* strains PAO1 and PA14. *J. Bacteriol*. **186,** 3270–3273.

Burns, J.C. and Globé, M.P. (2004). Kawasaki syndrome. *Lancet* **364,** 533–544.

Butler, T. (1983). *Plague and Other Yersinia Infection*. New York: Plenum Press.

Carnoy, C. and Simonet, M. (1999). *Yersinia pseudotuberculosis* superantigenic toxins. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins*. Alouf, J.E. and Freen J. H. (eds). London: Academic Press, pp. 611–622.

Carnoy, C., Mullet, C., Müller-Alouf, H., Leteutre, E. and Simonet, M. (2000). Superantigen YPMa exacerbates the virulence of *Yersinia Pseudotuberculosis* in mice. Infect Immun. 68, 2553-2559.

Carnoy, C., Floquet, S., Marceau, M., Sebbane, F., Haentjens-Herwegh, S., Devalckenaere, A. and Simonet, M. (2002). The superantigen gene *ypm* is located in an unstable chromosomal locus of *Yersinia pseudotuberculosis. J. Bacteriol*. **184,** 4489–4499.

Carnoy, C., Loiez, C., Faveeuw, C., Grangette, C., Desreumaux, P. and Simonet, M. (2003). Impact of the *Yersinia pseudotuberculosis*-derived mitogen (YPM) on the murine immune system. *Adv. Exp. Med. Biol*. **529,** 133–135.

Chain, P.S., Carniel, E., Larimer, F.W., Lamerdin, J., Stoutland, P.O., Regala, W.M., Georgescu, A.M., Vergez, L.M., Land, M.L., Motin, V.L., Brubaker, R.R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J.M., Derbise, A., Hauser, L.J. and Garcia, E. (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis. Proc. Natl. Acad. Sci. USA* **101,** 13826–13831.

Cheong, H.I., Choi, E.H., Ha, I.S., Lee, H.J. and Choi, Y. (1995). Acute renal failure associated with *Yersinia pseudotuberculosis* infection. *Nephron* **70,** 319–323.

Collyn, F., Fukushima, H., Carnoy, C., Simonet, M. and Vincent, P. (2005). Linkage of the horizontally-acquired *ypm* and *pil* genes in *Yersinia pseudotuberculosis. Infect. Immun*. **73**, 2556–2558.

Deng, W., Burland, V., Plunkett, G., 3rd, Boutin, A., Mayhew, G.F., Liss, P., Perna, N.T., Rose, D.J., Mau, B., Zhou, S., Schwartz, D.C., Fetherston, J.D., Lindler, L.E., Brubaker, R.R., Plano, G.V., Straley, S.C., McDonough, K.A., Nilles, M.L., Matson, J.S., Blattner, F.R. and Perry, R.D. (2002). Genome sequence of *Yersinia pestis* KIM. *J. Bacteriol*. **184,** 4601–4611.

Donadini, R., Liew, C.W., Kwan, A.H.Y., Mackay, J.P. and Fields, B.A. (2004). Crystal and solution structures of a superantigen from *Yersinia pseudotuberculosis* reveal a jelly-roll fold. *Structure* **12,** 145–156.

Donnelly, G.A., Lu, J., Takeda, T. and McKay, D.M. (1999). Colonic epithelial physiology is altered in response to the bacterial superantigen *Yersinia pseudotuberculosis* mitogen. *J. Infect. Dis*. **180,** 1590–1596.

Fukushima, H. and Gomyoda, M. (1991). Intestinal carriage of *Yersinia pseudotuberculosis* by wild birds and mammals in Japan. *Appl. Environ. Microbiol*. **57,** 1152–1155.

Fukushima, H., Matsuda, Y., Seki, R., Tsubokura, M., Takeda, N., Shubin, F.N., Paik, I.K. and Zheng, X.B. (2001). Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen *Yersinia pseudotuberculosis*-derived mitogen, and the high-pathogenicity island among *Yersinia pseudotuberculosis* strains. *J. Clin. Microbiol*. **39,** 3541–3547.

Gonzalez, M.D., Lichtensteiger, C.A., Caughlan, R. and Vimr, E.R. (2002). Conserved filamentous prophage in *Escherichia coli* O18:K1:H7 and *Yersinia pestis* biovar orientalis. *J. Bacteriol*. **184,** 6050–6055.

Homewood, R., Gibbons, C.P., Richards, D., Lewis, A., Duane, P.D. and Griffiths, A.P. (2003). Ileitis due to *Yersinia pseudotuberculosis* in Crohn's disease. *J. Infect*. **47,** 328–332.

Hugot, J.P., Alberti, C., Berrebi, D., Bingen, E. and Cezard, J.P. (2003). Crohn's disease: the cold chain hypothesis. *Lancet* **362,** 2012–2015.

Inoue, M., Nakashima, H., Ueba, O., Ishida, T., Date, H., Kobashi, S., Takagi, K., Nishu, T. and Tsubokura, M. (1984). Community outbreak of *Yersinia pseudotuberculosis. Microbiol. Immunol*. **28,** 883–891.

Ito, Y., Abe, J., Yoshino, K., Takeda, T. and Kohsaka, T. (1995). Sequence analysis of the gene for a novel superantigen produced by *Yersinia pseudotuberculosis* and expression of the recombinant protein. *J. Immunol*. **154,** 5896–5906.

Ito, Y., Seprényi, G., Abe, J. and Kohsaka, T. (1999). Analysis of functional regions of YPM, a superantigen derived from Gram-negative bacteria. *Eur. J. Biochem*. **263,** 326–337.

Jalava, K., Hallanvuo, S., Nakari, U.M., Ruutu, P., Kela, E., Heinasmaki, T., Siitonen, A. and Nuorti, J.P. (2004). Multiple outbreaks of *Yersinia pseudotuberculosis* infections in Finland. *J. Clin. Microbiol*. **42,** 2789–2791.

Kano, H., Ito, Y., Matsuoka, K., Nakajima, T., Iwata, T., Kohsaka, T., Saito, H. and Abe, J. (2004). Critical role of T cell migration in bacterial superantigen-mediated shock in mice. *Clin. Immunol*. **110,** 159–171.

Kawasaki, T. (1967). Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children. *Jpn. J. Allergy* **16,** 178–222.

Kawasaki, T., Kosaki, F., Okawa, S., Shigematsu, I. and Yanagawa, H. (1974). A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. *Pediatrics* **54,** 271–276.

Konishi, N., Baba, K., Abe, J., Maruko, T., Waki, K., Takeda, N. and Tanaka, M. (1997). A case of Kawasaki disease with coronary artery aneurysms documenting *Yersinia pseudotuberculosis* infection. *Acta Paediatr*. **86,** 661–664.

Llewelyn, M. and Cohen, J. (2002). Superantigens: microbial agents that corrupt immunity. *Lancet Infect. Dis*. **2,** 156–162.

Lu, J., Wang, A., Ansari, S., Hershberg, R.M. and McKay, D.M. (2003). Colonic bacterial superantigens evoke an inflammatory response and exaggerate disease in mice recovering from colitis. *Gastroenterology* **125,** 1785–1795.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248,** 705–711.

Mindich, L., Qiao, X., Qiao, J., Onodera, S., Romantschuk, M. and Hoogstraten, D. (1999). Isolation of additional bacteriophages with genomes of segmented double-stranded RNA. *J. Bacteriol*. **181,** 4505–4508.

Miyoshi-Akiyama, T., Imanishi, K. and Uchiyama, T. (1993). Purification and partial characterization of a product from *Yersinia pseudotuberculosis* with the ability to activate human T cells. *Infect. Immun*. **61,** 3922–3927.

Miyoshi-Akiyama, T., Abe, A., Kato, H., Kawahara, K., Narimatsu, H. and Uchiyama, T. (1995). DNA sequencing of the gene encoding a bacterial superantigen, *Yersinia pseudotuberculosis*-derived mitogen (YPM) and characterization of the gene product, cloned YPM. *J. Immunol*. **154,** 5228–5234.

Miyoshi-Akiyama, T., Fujimaki, W., Yan, X.J., Yagi, J., Imanishi, K., Kato, H., Tomonari, K. and Uchiyama, T. (1997). Identification of murine T cells reactive with the bacterial superantigen *Yersinia pseudotuberculosis*-derived mitogen (YPM) and factors involved in YPM-induced toxicity in mice. *Microbiol. Immunol*. **41,** 345–352.

Müller-Alouf, H., Proft, T., Zollner, T.M., Gerlach, D., Champagne, E., Desreumaux, P., Fitting, C., Geoffroy-Fauvet, C., Alouf, J.E. and Cavaillon, J.M. (2001). Pyrogenicity and cytokine-inducing properties of *Streptococcus pyogenes* superantigens: comparative study of streptococcal mitogenic exotoxin Z and pyrogenic exotoxin A. *Infect. Immun*. **69,** 4141–4145.

Müller-Alouf, H., Carnoy, C., Simonet, M. and Alouf, J.E. (2001). Superantigen bacterial toxins: state of the art. *Toxicon* **39,** 1691–1701.

Nakano, T., Kawaguchi, H., Nakao, K., Maruyama, T., Kamiya, H. and Sakurai, M. (1989). Two outbreaks of *Yersinia pseudotuberculosis* 5a infection in Japan. *Scand. J. Infect. Dis*. **21,** 175–179.

Nuorti, J.P., Niskanen, T., Hallanvuo, S., Mikkola, J., Kela, E., Hatakka, M., Fredriksson-Ahomaa, M., Lyytikainen, O., Siitonen, A., Korkeala, H. and Ruutu, P. (2004). A widespread outbreak of *Yersinia pseudotuberculosis* O:3 infection from iceberg lettuce. *J. Infect. Dis*. **189,** 766–774.

Palmer, L.E., Hobbie, S., Galan, J.E. and Bliska, J.B. (1998). YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF-alpha production and down-regulation of the MAP kinases p38 and JNK. *Mol. Microbiol*. **27,** 953–965.

Parkhill, J., Wren, B.W., Thomson, N.R., Titball, R.W., Holden, M.T., Prentice, M.B., Sebaihia, M., James, K.D., Churcher, C., Mungall, K.L., Baker, S., Basham, D., Bentley, S.D., Brooks, K., Cerdeno-Tarraga, A.M., Chillingworth, T., Cronin, A., Davies, R.M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A.V., Leather, S., Moule, S., Oyston, P.C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S. and Barrell, B.G. (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413,** 523–527.

Pebody, R., Leino, T., Holmström, P., Ruutu, P. and Vuento, R. (1997). Outbreak of *Yersinia pseudotuberculosis* infection in central Finland. www.eurosurveillance.org/ew/1997/970918.asp.

Petersson, K., Forsberg, G. and Walse, B. (2004). Interplay between superantigens and immunoreceptors. *Scand. J. Immunol*. **59,** 345–355.

Ramamurthy, T., Yoshino, K., Abe, J., Ikeda, N. and Takeda, T. (1997). Purification, characterization, and cloning of a novel variant of the superantigen *Yersinia pseudotuberculosis*-derived mitogen. *FEBS Lett*. **413,** 174–176.

Sato, K., Ouchi, K. and Taki, M. (1983). *Yersinia pseudotuberculosis* infection in children, resembling Izumi fever and Kawasaki syndrome. *Pediatr. Infect. Dis*. **2,** 123–126.

Schesser, K., Spiik, A.K., Dukuzumuremyi, J.M., Neurath, M.F., Pettersson, S. and Wolf-Watz, H. (1998). The *yopJ* locus is required for *Yersinia*-mediated inhibition of NF-kappaB activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol. Microbiol*. **28,** 1067–1079.

Seprényi, G., Ito, Y. and Kohsaka, T. (1999). Generated single-point mutations can considerably dismantle the lymphocyte overstimulation induced by *Yersinia pseudotuberculosis* superantigen. *Cell. Immunol*. **192,** 96–106.

Simonet, M.L. (1999). Enterobacteria in reactive arthritis: *Yersinia, Shigella,* and *Salmonella. Rev. Rhum. Engl. Ed*. **66,** 14S–18S.

Somov, G.P. and Martinevsky, I.L. (1973). New facts about pseudotuberculosis in the USSR. *Contrib. Microbiol. Immunol*. **2,** 214–216.

Song, Y., Tong, Z., Wang, J., Wang, L., Guo, Z., Han, Y., Zhang, J., Pei, D., Zhou, D., Qin, H., Pang, X., Zhai, J., Li, M., Cui, B., Qi, Z., Jin, L., Dai, R., Chen, F., Li, S., Ye, C., Du, Z., Lin, W., Yu, J., Yang, H., Huang, P., and Yang, R. (2004). Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. DNA Res. 11, 179-197.

Stuart, P.M. and Woodward, J.G. (1992). *Yersinia enterocolitica* produces superantigenic activity. *J. Immunol*. **148,** 225–233.

Stuart, P.M., Munn, R.K., DeMoll, E. and Woodward, J.G. (1995). Characterization of human T cell responses to *Yersinia enterocolitica* superantigen. *Hum. Immunol*. **43,** 269–275.

Takeda, N., Usami, I., Fujita, A., Baba, K. and Tanaka, M. (1991). Renal complications of *Yersinia pseudotuberculosis* infection in children. *Contrib. Microbiol. Immunol*. **12,** 301–306.

Tertti, R., Granfors, K., Lehtonen, O.P., Mertsola, J., Makela, A.L., Valimaki, I., Hanninen, P. and Toivanen, A. (1984). An outbreak of *Yersinia pseudotuberculosis* infection. *J. Infect. Dis*. **149,** 245–250.

Tertti, R., Vuento, R., Mikkola, P., Granfors, K., Makela, A.L. and Toivanen, A. (1989). Clinical manifestations of *Yersinia pseudotuberculosis* infection in children. *Eur. J. Clin. Microbiol. Infect. Dis*. **8,** 587–591.

Treacher, D.F. and Jewell, D.P. (1985). *Yersinia* colitis associated with Crohn's disease. *Postgrad. Med. J*. **61,** 173–174.

Uchiyama, T., Miyoshi-Akiyama, T., Kato, H., Fujimaki, W., Imanishi, K. and Yan, X.J. (1993). Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* isolated from patients manifesting acute and systemic symptoms. *J. Immunol*. **151,** 4407–4413.

Waldor, M.K. and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272,** 1910–1914.

Yagi, J., Dianzani, U., Kato, H., Okamoto, T., Katsurada, T., Buonfiglio, D., Miyoshi-Akiyama, T. and Uchiyama, T. (1999). Identification of a new type of invariant V $\alpha$ $14^+$ T cells and responsiveness to a superantigen, *Yersinia pseudotuberculosis*-derived mitogen. *J. Immunol*. **163,** 3083–3091.

Yoshino, K., Abe, J., Murata, H., Takao, T., Kohsaka, T., Shimonishi, Y. and Takeda, T. (1994). Purification and characterization of a novel superantigen produced by a clinical isolate of *Yersinia pseudotuberculosis. FEBS Lett*. **356,** 141–144.

Yoshino, K., Ramamurthy, T., Nair, G.B., Fukushima, H., Ohtomo, Y., Takeda, N., Kaneko, S. and Takeda, T. (1995). Geographical heterogeneity between Far East and Europe in prevalence of ypm gene encoding the novel superantigen among *Yersinia pseudotuberculosis* strains. *J. Clin. Microbiol*. **33,** 3356–3358.

Yoshino, K., Takao, T., Ishibashi, M., Samejima, Y., Shimonishi, Y. and Takeda, T. (1996). Identification of the functional region on the superantigen *Yersinia pseudotuberculosis*-derived mitogen responsible for induction of lymphocyte proliferation by using synthetic peptides. *FEBS Lett* **390,** 196–198.

CHAPTER

# 53

# Comparative three-dimensional structure of bacterial superantigenic toxins

*Matthew D. Baker and K. Ravi Acharya*

## INTRODUCTION

From a historical perspective, the discovery and characterization of bacterial superantigens (SAgs) is heavily indebted to the structural techniques used to probe their many functions. From the first three-dimensional structure of a bacterial superantigen (Swaminathan *et al*., 1992) to the complexes of these toxins with their receptors (Jardetzky *et al*., 1994; Kim *et al*., 1994; Fields *et al*., 1996; Li *et al*., 1998a, 2001; Petersson *et al*., 2001), the complete archaeology of these molecules has been mapped and a wealth of information has been obtained. The ability to assign specific functions to discrete regions of the toxin and to assess the roles of individual amino acid residues by a combination of mutagenesis and structural techniques has enabled detailed comparisons of the entire bacterial superantigen family of proteins. In general, the nature of these toxins makes them particularly suitable for structural analysis by x-ray crystallography. Crystallization requires large quantities of protein, and these toxins can be expressed and purified in high quantities from their native bacteria (Chu *et al*., 1966; Avena and Bergdoll, 1967; Robern *et al*., 1975; Ende *et al*., 1983; Reynolds *et al*., 1988; Brehm *et al*., 1990) or in a recombinant form (Munson *et al*., 1998; Proft *et al*., 1999; Sundberg and Jardetzky, 1999; Donadini *et al*., 2004; Zhao *et al*., 2004). Crystallization also dictates that numerous trials may need to be carried out at different temperatures over wide pH ranges and at various concentrations of salts and precipitants. Luckily, these purified toxins are very stable, being highly resistant to proteases, denaturation from heat, and extreme pH (Marrack and Kappler, 1990) characteristics that have proved to be vital for structural studies.

Superantigens are powerful T cell stimulatory molecules produced primarily by *Staphylococcus aureus* and *Streptococcus pyogenes*. The action of these toxins as SAgs can be attributed to their ability to cross-link MHC class II molecules and T cell receptors to form a trimolecular complex (Marrack and Kappler, 1990). In contrast to conventional antigens, they are not processed internally by antigen presenting cells (APC) and act as an intact fully folded protein rather than an antigenic peptide located in the peptide-binding groove of the MHC class II molecule. Structural evidence shows SAgs bind to APCs on the outside of MHC class II molecules (Jardetzky *et al*., 1994; Kim *et al*., 1994; Li *et al*., 2001; Petersson, 2001) and to T cells via the external face of the T cell receptor (TCR) $V_\beta$ element (Li *et al*., 1998a; Fields *et al*., 1996) (Figure 53.1).

The number of known bacterial proteins with superantigenic properties and/or high homology with known SAgs has grown considerably over the last decade. Staphylococcal enterotoxins (SEs), A, B, C1–3, D, E, H, I, J; toxic shock syndrome toxin-1 (TSST-1), the streptococcal pyrogenic exotoxins (Spes) A, C, H; and streptococcal mitogenic exotoxin SME-$Z_2$ and streptococcal superantigen (SSA) are the most well studied bacterial SAgs to date (for recent reviews see Papageorgiou and Acharya, 2000; Baker and Acharya 2003, 2004). Other pathogens, such as *Mycoplasma arthritidis* and *Yersinia enterocolitica* have also been shown to secrete superantigenic proteins (Cole *et al*., 1996), and

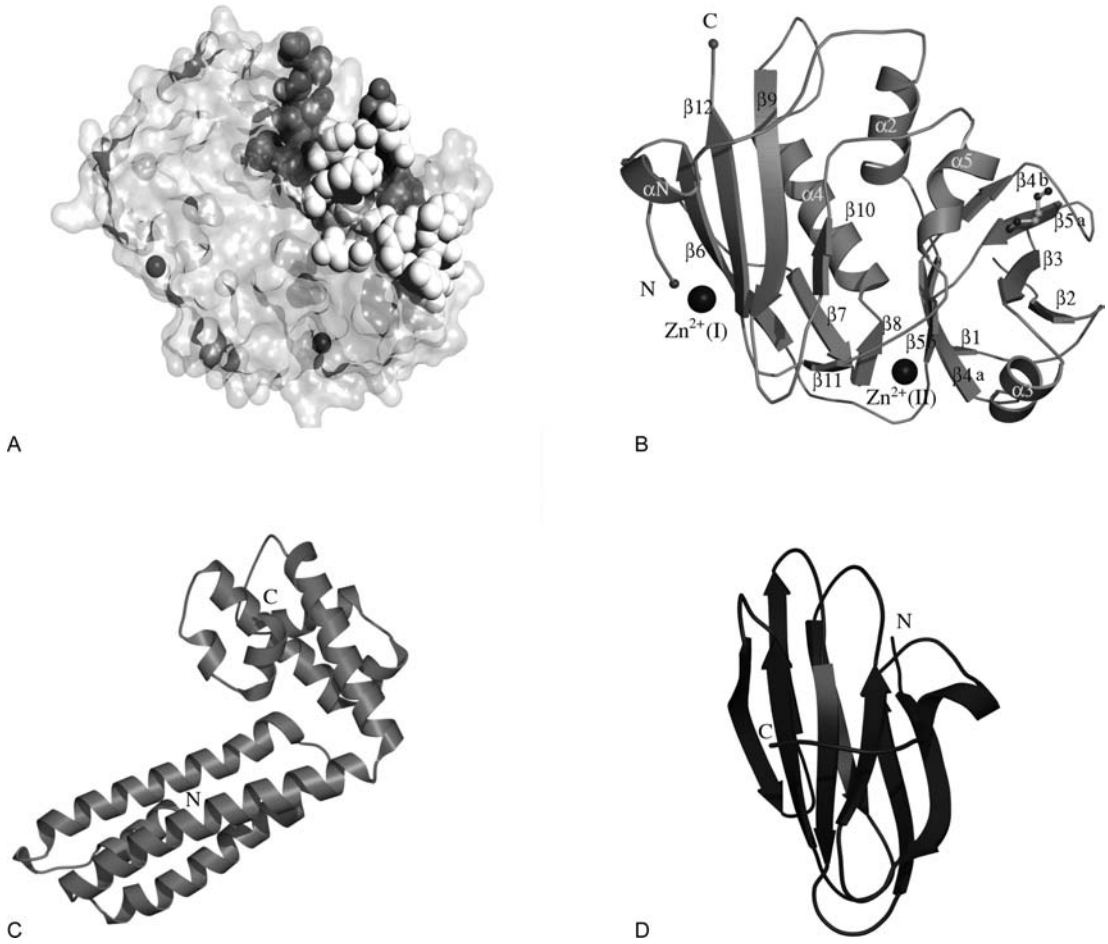



**FIGURE 53.1** (A) Surface representation of a typical staphylococcal/streptococcal superantigen with TCR binding region (dark grey) and MHC class II binding region (white) indicated; (B) Ribbon diagram of SEA representative of the common structural features of the staphylococcal and streptococcal superantigen family. Spheres represent the positions of the two zinc sites. The cysteine residues that form the disulphide loop are shown in ball-and-stick representation and all secondary structure elements are labeled; (C) The crystal structure of MAM (helical structure); (D) The crystal structure of YPM with a β-barrel motif.

the characterization of these toxins has recently been helped by the elucidation of their crystal structures (Donadini *et al*., 2004; Zhao *et al*., 2004).

Staphylococcal and streptococcal SAgs can be grouped into four subfamilies based on amino acid sequence and structure (Williams *et al*., 2000; Thomas *et al*., 2004). The first group is made up of the staphylococcal enterotoxins SEA, SED, SEE, SEH, SEI, and SEJ. Both staphylococcal and streptococcal toxins form the second group, which is comprised of SEB, SEC1-3, SpeA1-3, SSA, and SEG. The third group contains streptococcal pyrogenic and mitogenic toxins; SpeC, SpeJ, SpeG, SpeH, SME-Z, SME-$Z_2$, and streptococcus dysgalactiae-derived mitogen (SDM). The staphylococcal superantigen–like proteins (SSL, previously named as SET) and TSST-1 form the fourth group. Mycoplasma arthritidis mitogen (MAM) and Yersinia pseudotuberculosis mitogen (YPM) have no sequence homology to the rest of the bacterial SAgs and cannot be grouped with any of these subfamilies (Cole *et al*., 1996; Ito *et al*., 1999). In addition, the bounty of information from genomics initiatives (Kuroda *et al*., 2001; Ferretti *et al*., 2001) has led to the discovery of new putative SAgs based on amino acid sequence alignment; examples include SEQ (Orwin *et al*., 2002), SER (Omoe *et al*., 2003), SpeL, and SpeM (Proft *et al*., 2004). Due to the growing number of potential SAgs, the International Nomenclature Committee for Staphylococcal Superantigens has proposed a new procedure for the naming of newly described SAgs and the renaming of some previously described SAgs (Lina *et al*., 2004).

On comparison of the three-dimensional structures of SAgs (see Papageorgiou and Acharya, 2000 and references therein), a two-domain architecture is apparent. The two domains (N- and C-terminal domains) are separated by a long, solvent-accessible α-helix, which

spans the center of the molecule (Figure 53.1). The N-terminal domain is characterized by the presence of hydrophobic residues in its solvent-exposed regions and has considerable structural similarity to the oligosaccharide/oligonucleotide binding–fold (OB-fold). Despite this similarity, SAgs have not been shown to bind DNA or recognize carbohydrates. The C-terminal domain comprises a four-stranded β-sheet capped by a central α-helix and resembles the β-grasp motif. Other features common to a significant number of family members include a highly flexible disulphide loop, which is located in the N-terminal domain (Figure 53.1 and Table 53.1). This flexible loop is thought to be involved in the emetic properties of the staphylococcal and streptococcal toxins, as substitution of the two cysteine residues that form this disulphide loop to alanine abolishes the emetic activity in SEC1 (Hovde *et al*., 1994). The corresponding cysteine residues in SpeA1 have also been shown to be involved in T cell stimulation, as their mutation significantly reduces the ability of the toxin to stimulate certain populations of T cells (Kline and Collins, 1997). The recent structures of the staphylococcal superantigen-like proteins SSL5 and SSL7 have revealed that the common architecture is preserved among these related groups of proteins (Arcus *et al*., 2002; Al-shangiti *et al*., 2004). SSL5 is most closely related to TSST-1, but shows two notable structural differences from other members of the superantigen family. First, the β6 and β7 strands of the C-terminal domain are extended such that the β6–β7 loop extends significantly from the molecular surface of the molecule. Second, there is a widespread positive charge over the surface of the protein, particularly concentrated at the central α-helix and the outer face on the N-terminal domain. SSL7 is most closely related to SpeC, but like SSL5 shows no superantigenic activity (Al-Shangiti *et al*., 2004). SSL7 is also thought to be able to form dimers via the interaction of the β-grasp domains of two monomers to form an intermolecular β-sandwich. At present, it is not known if the features of either of these proteins have any biological significance.

Two recently obtained crystal structures for Mycoplasma arthritidis mitogen (MAM) and Yersinia pseudotuberculosis mitogen a (YPMa) have extended the topology of the family further (Zhao *et al*., 2004; Donadini *et al*., 2004). The crystal structure of MAM reveals the toxin to have an entirely different architecture to the staphylococcal and streptococcal toxins (Figure 53.1). MAM is composed of two α-helical domains forming an L-shape. The N-terminal domain consists of four α-helices and a 25 residue long N-terminal loop that wraps around the four-helix bundle. The C-terminal contains six α-helices. Yersinia pseudotuberculosis mitogen a (YPMa) is much smaller than the classical staphylococcal and streptococcal toxins with a molecular mass of 14 kDa. It consists of a jelly-roll fold comprising two β-sheets, each containing four anti-parallel strands (Donadini *et al*., 2004). The structure is unlike any known SAgs, being structurally similar to viral capsid proteins and members of the tumor necrosis factor superfamily (Figure 53.1).

## BINDING TO MHC CLASS II MOLECULES

Structural analyses using x-ray crystallography have shown that MHC class II molecules possess two distinct binding sites for the classical staphylococcal and streptococcal SAgs (Papageorgiou and Acharya, 2000). The first, a low-affinity binding site (referred to as the generic site) is located on the α-chain of the MHC class II molecule and the second, a high-affinity (about 100 times higher affinity than the generic site), zinc-dependent site is located on the β-chain (Figures 53.2 and 53.3). The structures of SEB and TSST-1 in complex with HLA-DR1 via the low-affinity site (Jardetzky *et al*., 1994; Kim *et al*., 1994), SpeC in complex with HLA-DR2 (Li *et al*., 2001), and SEH in complex with HLA-DR1 (Petersson, 2001) via the high-affinity site have yielded a great deal of information about the binding of SAgs to MHC class II molecules. It appears that each superantigen binds to different alleles of class II molecules to varying degrees. While the majority of the SAgs including TSST-1, SEB, and MAM bind preferentially to HLA-DR alleles, SAgs such as SEC, SpeA, and SSA bind predominantly to HLA-DQ alleles (Sundberg and Jardetzky, 1999; Etongue-Mayer *et al*., 2002).

It is apparent that both SEB · and TSST-1 · HLA-DR1 complexes have similar binding modes to the DR1 α-chain, with the solvent-exposed, hydrophobic core at the N-terminal domain of the toxin playing an important role. Similar hydrophobic ridge regions exist in several other SAgs and form the generic MHC class II binding site. However, in the case of TSST-1, additional contacts with the peptide antigen were also present (Kim *et al*., 1994). Indeed, truncating the C-terminal end of the peptide dramatically affects TSST-1 binding to murine I-Ab (Wen *et al*., 1997).

Many members of the superantigen family (except SEB, TSST-1, SSA, SET1-5, and YPM) possess either one or two zinc binding sites (Papageorgiou and Acharya, 2000) (for details see Table 53.1, Figures 53.2 and 53.3). Zinc ions have been shown to be important for the recognition of the MHC class II β-chain. Mutational and structural analyses have identified a high-affinity zinc-binding site in SEA at the C-terminal domain with a $K_d$ of 100 nM for DR1 recognition (Fraser *et al*., 1992).

**TABLE 53.1** The bacterial superantigen family

| Superantigen | Mr (kDa) | MHC-II generic site | Zinc usage | Dimer formation | Disulphide loop |
|---|---|---|---|---|---|
| ***S. aureus*** | | | | | |
| SEA | 27.1 | Yes | High-affinity site<br>Low-affinity site | Yes | Yes |
| SEB | 28.4 | Yes | No | | Yes |
| SEC1-3 | 27.5 | Yes | | Yes | Yes |
| SED | 26.3 | Yes | High-affinity or dimer?<br>Low-affinity site | Yes | Yes |
| SEE | 26.4 | Yes | High or low | | Yes |
| SEG | 27 | | No | | Yes |
| SEH | 25.2 | No generic site | Single | | Yes |
| SEI | 24.9 | | No | | No |
| TSST-1 | 22 | Yes | No | | No |
| SSL1-11 | ~29 | No | No | | No |
| ***S. pyogenes*** | | | | | |
| SpeA | 25.7 | Yes | Single | Yes | Yes |
| SpeC | 24.4 | No generic site | Single | Yes | No |
| SpeG | 24.6 | No generic site | #single | | |
| SpeH | 23.6 | No generic site | single | | Yes |
| SpeJ | 24.7 | No generic site | Single | Yes | |
| SME-Z | 24.3 | No generic site | #single | | No |
| SME-Z2 | 24.2 | No generic site | single | | No |
| SMEZ3/SPEX | | No generic site | #single | | |
| SSA | 26.9 | Yes | No | | Yes |
| ***S. dysgalactiae*** | | | | | |
| SDM | 25 | | #single | | No |
| ***Yersinia pseudotuberculosis*** | | | | | |
| YPM | 14.5 | | | | – |
| ***Mycoplasma arthritidis*** | | | | | |
| MAM | 27 | | | Yes | – |

#Proposed residues based on sequence alignment

In comparison, the zinc-independent generic site at the N-terminal domain has considerably lower affinity ($K_d$ of 10 μM) for class II binding. If the two binding sites coexist, SEA shows a $K_d$ of 13 nM. Mutation of residues in either of these sites results in a toxin unable to induce cytokine expression in peripheral blood mononuclear cells (PBMC) (Abrahmsen *et al*., 1995). It would seem that SEA possesses two distinct MHC class II binding sites, which may enable it to form trimeric SEA·MHC·SEA complex, a phenomenon that has been observed in solution experiments (Tiedemann *et al*., 1995). Similar arguments can be put forward for SEE, as both SEA and SEE possess identical zinc ligands.

The high-affinity site of SEA is not present in SEC or SpeA. Instead, a new zinc binding site with somewhat lower affinity compared with the high-affinity zinc binding described above (the estimated dissociation constant for the zinc ion in SEC2 is less than 1 μM) was identified at the N-terminal domain, which also appears to be important for MHC class II binding (Figures 53.2 and 53.3) (Papageorgiou *et al*., 1999). This site is known as the secondary zinc-binding site. In SED (Sundstrom *et al*., 1996) and SpeC (Roussel *et al*., 1997), the dimerization mechanism varies slightly. SED can form zinc-dependent homodimers, while SpeC forms zinc-independent homodimers. Both SED and SpeC bind to the β-chain of MHC class II molecules through a zinc-mediated mechanism similar to that of SEA and formation of trimers and/or tetramers is a possibility. A similar binding mechanism has been proposed for SEH, which also lacks a generic MHC class II binding site (Hakansson *et al*., 2000).

The crystal structures of SpeC in complex with HLA-DR2 (Li *et al*., 2001) and SEH in complex with HLA-DR1 (Petersson, 2001) via a high-affinity, zinc-dependent site

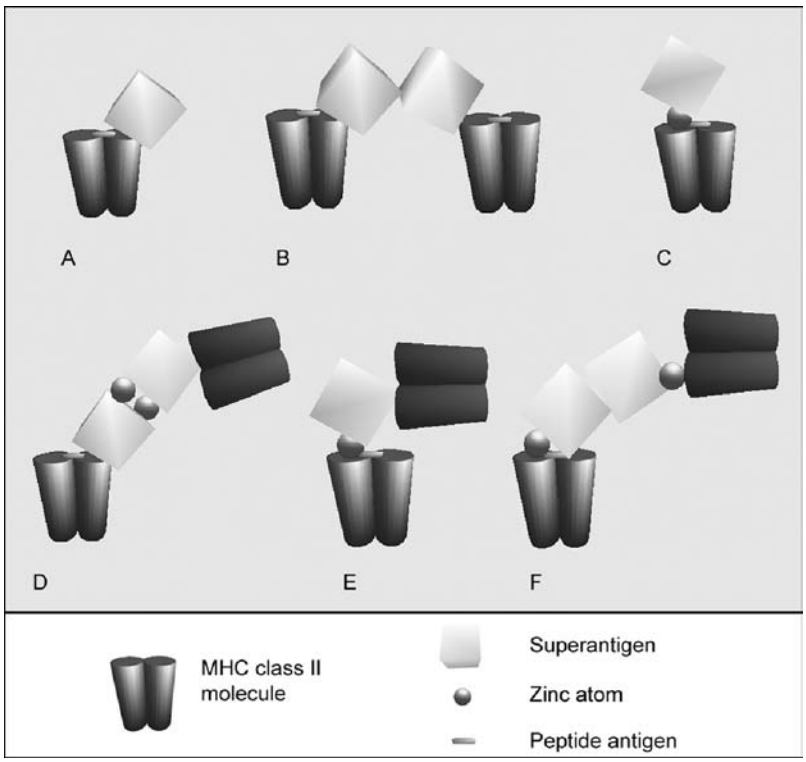



**FIGURE 53.2** Schematic diagram illustrating the multiple modes by which SAgs can interact with MHC class II molecules; (A) Interaction with a single MHC class II molecule via the generic binding site on the α chain; e.g., SEB and TSST-1; (B) Interaction of a non-zinc linked superantigen dimer with two separate MHC class II molecules via the α chain generic site; e.g., MAM; (C) Interaction of a superantigen with MHC class II β chain via a high-affinity zinc site; e.g., SEH and SpeC; (D) A zinc-linked superantigen dimer interacting with two MHC class II molecules via their α chain generic binding sites; e.g., SE; (E) Interaction of a single superantigen with two MHC class II molecules—both via the generic site (α chain) and the high-affinity zinc site on the β chain; e.g., SEA and SEC2; (F) Dimeric superantigen bound to two MHC class II molecules via their high-affinity zinc sites; e.g., SpeC and SpeA1.

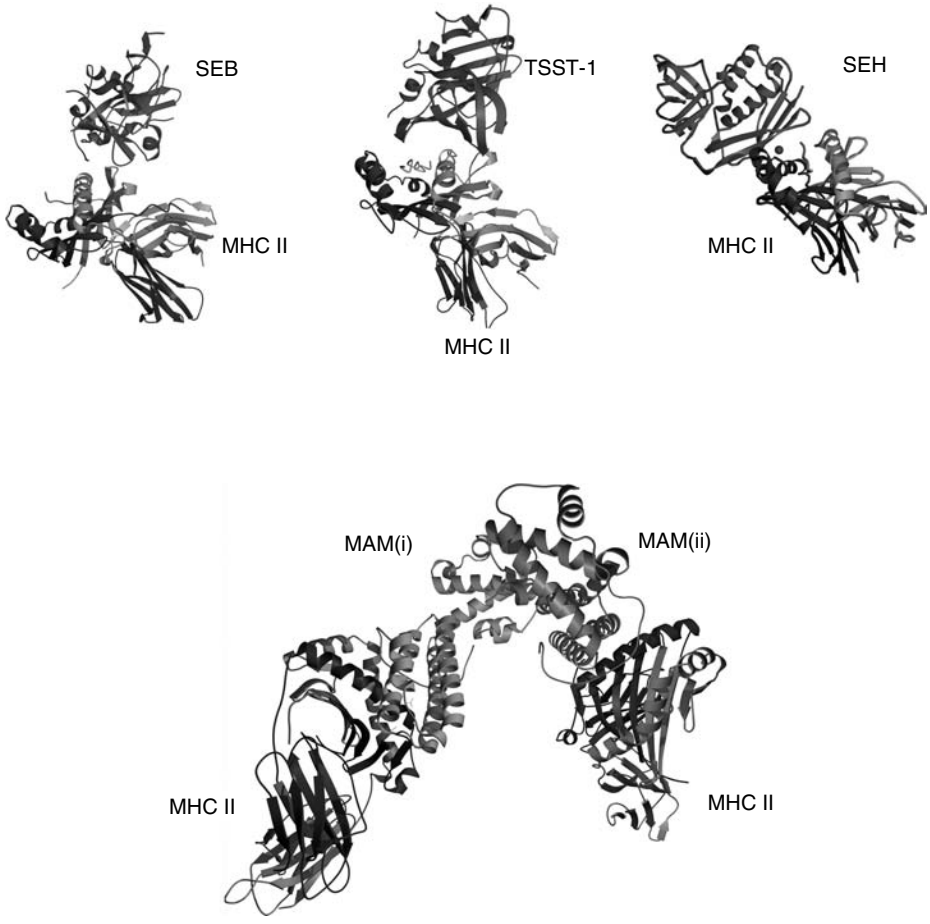



**FIGURE 53.3** Crystal structures of SAgs in complex with MHC class II molecules. **Top left:** SEB in complex with MHC class II molecule (HLA-DR1) via the generic site. **Top middle:** TSST-1 in complex with HLA-DR1 via the generic site. **Top right:** SEH in complex with HLA-DR1 via a high-affinity zinc site. **Bottom**: MAM dimer in complex with two HLA-DR1 molecules via a generic site.

have shed more light on the interactions of SAgs with MHC class II molecules (Figure 53.3). The interactions between both SAgs and their MHC class II molecules are governed by a bridging zinc ion, which tetrahedrally coordinates three ligands from the SpeC (His 167, His 201, and Asp 203) and two from SEH (His 206, Asp 208, and a water molecule) with one from the MHC class II β1 helix (His 81). Approximately ⅓ of the contact area between SpeC and MHC class II is taken up by antigenic peptide. Similarly, stability of the SEH–MHC class II complex is also achieved through interaction with the antigenic class II associated peptide, and in both cases a majority of the interactions with the antigenic peptide are via its backbone atoms. Hence, both the SpeC and SEH complexes have similar interactions with the antigenic peptide despite differences in peptide composition. This indicates that although the peptide plays an important role in the complex interaction, binding to MHC class II is not entirely peptide specific.

Zinc is also shown to play a role in the binding of SME-$Z_2$, SpeG, and SpeH to MHC class II molecules, as the binding of all three of these SAgs to LG-2 cells is significantly reduced by the addition of EDTA (Proft *et al*., 1999). The proposed zinc binding site in each of these SAgs is shown to be closest to that of SEA and SpeC, both of which have geometrically and spatially equivalent sites (Roussel *et al*., 1997; Proft *et al*., 1999). As the presence of the zinc-binding ligands suggest, all three of these SAgs bind to MHC class II molecules in a zinc-dependent fashion. Recently, a second zinc binding site was discovered in SEC2 (Papageorgiou *et al*., 2004). This binding site is located close to the generic MHC class II binding site and is postulated to serve as a site of dimerization for SEC2. If this is the case, the generic MHC class II site would be blocked by the dimer interface, and binding to MHC class II molecules would have to be mediated by the SEC2 primary zinc-binding site. This would result in SEC2 being able to bind to MHC class II molecules via a zinc-mediated dimer. SpeA1 and SpeC are also able to form zinc-independent homodimers. SpeA1 has recently been shown to exist in a disulphide linked dimeric form (Baker *et al*., 2004b) via cysteine residues located within the flexible disulphide loop, while the SpeC dimer is formed by using the surface that is usually used as a generic MHC class II binding site (Roussel *et al*., 1997). The crystal structure of SpeJ is also shown to bind to MHC class II molecules in a zinc-dependent fashion (Baker *et al*., 2004a).

The SSL proteins are unique among the superantigen family in that they do not bind to MHC class II molecules and do not appear to be superantigenic (Arcus *et al*., 2002; Al-Shangiti *et al*., 2004). Hence, the conserved amino acids appear to be playing a role in preserving the superantigen fold, and the large number of non-conservative substitutions within the generic MHC class II binding site mean that MHC II binding is unlikely.

Zinc was also thought to play a role in MAM binding to MHC class II molecules or in dimerization of the toxin (Etongue-Mayer *et al*., 2002; Langlois *et al*., 2003). However, the absence of zinc from the crystal structure of dimeric MAM in complex with HLA-DR1 (Zhao *et al*., 2004) supported no such role, and the exact role of zinc in the action of this novel superantigen requires further investigation.

Although the structure of MAM complexed with HLA-DR1 bears no resemblance to the classical staphylococcal and streptococcal SAgs, there are some similarities in the general mechanism of binding. Upon complex formation, there are no major structural changes at the interface between MAM and MHC class II molecules, which is consistent with the complexes of other bacterial SAgs with MHC class II. The MAM·HLA-DR1 complex is formed through contacts between the N-terminal domain of MAM and the several regions of HLA-DR1; specifically, the β1-β2 loop, the β3-β4 loop, and the α1 helix of the DR1 α chain; the β1 helix of the DR1 β chain; and the hemagglutinin (HA) peptide bound in the peptide binding groove of the HLA-DR1 molecule. The particular regions on the N-terminal domain of MAM that contact HLA-DR1 are α3- and α4-helices, which interact with the α1 helix of the DR1 α chain and both the β1-β2 loop and the β3-β4 loop of the DR1 α chain. The N-terminal loop of MAM also makes extensive contact with the HA peptide. Comparison of the binding of MAM to MHC class II molecules with other SAgs reveals that the MAM binding site on HLA-DR1 also overlaps with the generic binding site for staphylococcal and streptococcal SAgs. Indeed, SEB and TSST-1 have been shown to block MAM binding to MHC class II on THP-1 cells (Bernatchez *et al*., 1997). MAM binding is also abolished by superantigen binding to the high-affinity zinc site, as the MAM binding site on MHC class II also encompasses a region on the MHC β chain within which His 81 is located (Zhao *et al*., 2004). The binding mode of each of the two MAM monomers with a single HLA-DR1 is identical. MAM is shown to exist as both monomer and homodimer in solution, indicating that it may be able to act on MHC class II molecules as both monomer and dimer. The interaction between the two molecules does not involve the zinc ion and is formed by the C-terminal domain on one monomer sitting in the V-shaped cleft formed by both the N- and C-terminal domain of the second monomer (Figure 53.3).

Presently, the mechanism of YPM binding to MHC class II molecules is unknown. In the absence of a structure in complex with MHC class II and no homology to

the rest of the superantigen family, mutagenesis studies have been employed in the hope of shedding further light on this problem (Ito *et al*., 1999). However, the crystal structure of YPM has revealed that a majority of the mutations that affected activity were buried within the structure. Therefore, it is likely that these mutations caused conformational changes and/or misfolding of the protein. As such, it is imperative that further work be carried out in order to characterize the functional regions of this toxin.

It appears that there is an ever-broadening array of mechanisms by which SAgs can mediate binding with MHC class II molecules. This can be either through zinc-mediated interaction or via the generic site or as a homodimer (which in turn can be either zinc and non-zinc mediated) or involving a combination of all three mechanisms.

## BINDING TO THE T CELL RECEPTOR

The interaction of SAgs with T cell receptors is seemingly a much simpler affair; SAgs were thought to bind exclusively to TCR via the $V_\beta$ element through a universally similar mechanism in which the binding is mediated by interactions between the side chains of the superantigen and the $V_\beta$ backbone atoms. The net result is that each superantigen expands T cells bearing certain $V_\beta$ elements, while others are excluded (Kappler *et al*., 1989).

The characterization of a superantigen TCR binding region reveals a similar framework with specific differences that may reflect differences in $V_\beta$ specificity. The TCR binding site involves the shallow cavity between the two domains of the molecule. In SEB this cavity is formed by residues 22–33 (mostly α2 helix), 55–61 (β2-β3 loop), 87–92 (β4 strand and β4-β5 loop), 112 (β5 strand), and 210–214 (α5 helix) (Swaminathan *et al*., 1992) (Figure 53.1), and equivalent sites for SEC and SEA have also been suggested using the structure-based sequence alignment. Crystal structures of SAgs with various TCR $V_\beta$ elements have been elucidated (Fields *et al*., 1996; Li *et al*., 1998a; Sundberg *et al*., 2002). The structures of SEB, SEC2, SEC3 appeared to back up this simple binding mechanism with a majority of the contacts being made in the form of interactions between the side chains of the superantigen and backbone atoms of the TCR $V_\beta$ chain. For SEC2, SEC3, and SEB the main interactions are shown to be between the side chain atoms of the superantigen and complementary determining regions one and two (CDRs 1 and 2), and hypervariable region 4 (HV4) of the $V_\beta$ chain. Comparison of this TCR binding site (from SEC2/3) with the corresponding regions of SEA and SEB identifies an invariant asparagine residue (Asn23 in SEB/SECs; Asn25 in SEA) as being crucial for direct interactions with the TCR. Mutation of this residue in SEB results in the loss of T cell stimulation (Kappler *et al*., 1992). This residue is solvent exposed in SEA, SEB, and SEC, and is thought to have similar interactions in all the SEs. Alanine scanning mutagenesis of individual SEC3 residues involved in the stabilization of the SEC3/$V_\beta$ complex in the crystal structure (Fields *et al*., 1996; Leder *et al*., 1998) showed that Asn23, Tyr90, and Gln210 had the most effect on controlling binding to the TCR β-chain. Tyr90 and Gln210 are conserved among SEC1-3, SEB, and SpeA, and SSA has analogous residues Asn49, Tyr116, and Gln223 (Swaminathan *et al*., 1995; Stevens *et al*., 1996). The variance between SAgs with regard to TCR affinity and specificity can be accounted for by several residues unique to each particular superantigen, as well as any topological influence that each of these might have. For example, residue Tyr26 of SEC2 confers specificity between SEC1 and SEC2 via its interaction with Gly53 from the $V_\beta$ chain (Deringer *et al*., 1996) and is not conserved in SEA or SEB. Val91 of SEC2 is also implicated in TCR binding and is not conserved in SEA (Tyr94) or SEB (Tyr91) either (Tiedemann *et al*., 1995). It is possible that the switching of Val91 for a tyrosine residue in SEB may be responsible for its decreased affinity for the Vβ8.2 chain (Fields *et al*., 1996). SEA specificity for TCR is thought to be governed by Ser206, Asn207, and Thr21 (Swaminathan *et al*., 1995). Further evidence for this is provided by the exchange of residues 206 and 207 in SEA for the homologous residues in SEE. This causes a switch in the profile of $V_\beta$ elements on the responding T cells to change to that of the profile normally seen for T cells stimulated by SEE (Hudson *et al*., 1993). Ser206 and Asn207 in SEA correspond to Gln210 and Ser211, respectively, in both SEB and SEC2. Those residues that define the specificity of a superantigen for particular $V_\beta$ elements make the greatest energetic contribution to the overall stability of the $V_\beta$ superantigen complex (Li *et al*., 1998b), and as the above evidence shows, these residues are relatively few in number.

The crystal structure of SpeA1 in complex with mouse $V_\beta$ 8.2 and SpeC in complex with human $V_\beta$ 2.1 add extra dimensions to the apparently simple mechanism of TCR binding of SAgs (Sundberg *et al*., 2002). First, it is evident that SpeC binds to significantly more $V_\beta$ residues than the SEC2/3 or SEB and encompasses areas of the CDR1 and CDR3 loops of the TCR β chain. This increased number of contacts is thought to be due to the deeper and broader cleft between the N- and C-terminal domains of SpeC where the TCR binding site is located. In addition, SpeC forms numerous interactions with both main chain and side chain atoms of

the TCR $V_\beta$ chain. While interaction of SpeA1 with the TCR $V_\beta$ chain is similar to that of SEB (conserving the three hydrogen bonds seen between SEB and the TCR), it has an additional five hydrogen bonds compared to SEB. The differences in the TCR binding sites of these two SAgs and the interactions of amino acid side chains on both the superantigen and the TCR $V_\beta$ chain suggest that binding to TCR receptors is not merely one of simple conformational dependence (Fields *et al*., 1996; Li *et al*., 1998b). The crystal structure of SpeJ and subsequent mutagenesis work on its TCR binding site is shown to be very similar to that of SpeC (Baker *et al*., 2004a). However, SpeJ has also been shown to use its TCR binding site to form homodimers, an action that would occlude its ability to bind to TCR molecules. At present, the ramifications of this mode of dimeriztion for TCR binding by SpeJ are unclear.

The TCR binding site of TSST-1 is as yet not fully characterized. As it is structurally similar to SpeC and SpeJ, it is possible that it shares similar TCR binding features. The available mutagenesis data suggest that the TCR binding site of TSST-1 is located in the C-terminal domain on the long α2 helix and between the β7–β8 and α2–β9 loops as part of the α1 helix. In this regard, it is unique from the SEs (Acharya *et al*., 1994). Mitogenicity is lost either partially or completely by mutation of residues in the region 115–144. In particular, residues Tyr115, Glu132, His 135, Ile140, His 141, and Tyr144 are all of great importance for TSST-1 binding to TCR (Acharya *et al*., 1994; Deresiewicz *et al*., 1994). Mutation of these residues produces substantially fewer mitogenic toxins, yet they can still be recognized by a specific antibody, suggesting that the toxins produced are still intact (Deresiewicz *et al*., 1994).

Given the high degree of similarity between the SET proteins and TSST-1, it is somewhat surprising that they do not stimulate T cells in a $V_\beta$ restricted manner (Arcus *et al*., 2002). Closer examination of the structure of SSL5 reveals that amino acid conservation is restricted to those residues that confer structural integrity, particularly in and around the region joining the N- and C-terminal domains where the TCR binding site is located in TSST-1. Additional changes to specific surface residues further serve to change the nature of the protein, making it unable to interact with TCR molecules.

Both MAM and YPM are known to bind to the TCR $V_\beta$ chain (Cole *et al*., 1991; Abe *et al*., 1993; Uchiyama *et al*., 1993). Although their crystal structures have allowed some speculation as to how they may interact with TCRs, at present there is not enough data to fully characterize these interactions, and it will be interesting to see if these novel SAgs bind to TCR in a similar manner to the staphylococcal and streptococcal SAgs.

Structural biology has enabled us to characterize the TCR $V_\beta$ chain binding sites of several SAgs. While the interactions with TCR share a common core of residues, it is evident that specificity for particular $V_\beta$ elements comes from amino acid residues unique to each toxin. This in turn demonstrates that there are also multiple modes by which SAgs can interact with the TCR. First, there is a high-specificity binding mode involving many contacts by both backbone atoms and side chain atoms over an increased area, as demonstrated by SpeC. This mode has a high affinity for only a few TCR $V_\beta$ elements; second, there is a moderate specificity mode with fewer interactions over a reduced contact area. This mode has a modest affinity for a larger group of TCR $V_\beta$ elements, such as those seen in SpeA1. Thirdly, there is a promiscuous binding mode, such as is seen in SEB and SEC2, that binds to TCR $V_\beta$ chains in a simple conformation-dependent manner. A further complication to this mechanism was unearthed when it was discovered that SEH, in contrast to all other SAgs, stimulates T cells in a $V_\alpha$ specific fashion with a complete absence of any $V_\beta$ expansion (Petersson *et al*., 2003). It is postulated that SEH may bind to TCR in this way due to its presentation by MHC class II molecules via a zinc atom. Whether other SAgs that are presented to TCR by MHC class II in the same way can bind to $V_\alpha$ in the same fashion remains to be seen, and further investigation is essential. As more superantigen TCR complexes are characterized structurally, the complexities of the interactions that lead to each superantigen having a distinct $V_\beta$ (or $V_\alpha$) profile will be better understood.

## OTHER STRUCTURAL FEATURES AND IDIOSYNCRASIES

The ability of TSST-1 to cause systemic or localized symptoms in a site-dependent manner led to the proposal that it, in contrast to the other SEs, must be able to transverse the epithelial barrier (Schlievert *et al*., 2000). The mechanism by which it is able to do so could include either passive diffusion or cellular receptors. If a cellular receptor is required, then what is the structural evidence for such a binding site on TSST-1? Obvious structural differences between TSST-1 and the other SAgs include the lack of an α-helix in the C-terminal domain, the long N-terminal extension, and the absence of a disulphide loop. TSST-1 also has unique patches of hydrophobic and neutral residues on the front and rear of the β-barrel at the N-terminal

domain. Many of these features could combine to produce a receptor-specific binding site in order for TSST-1 to traverse epithelial cells and allow systemic shock.

The SET group of proteins are most closely related to TSST-1, yet have no superantigenic activity (Arcus *et al*., 2002). The structure of SSL5 reveals a widespread positive charge over the protein's surface, which is suggested to be the likely binding site for negatively charged binding partners. By modeling the sequences of several of the SSL proteins onto the structure of SSL5, it was found that the surface features were altered. This supports the hypothesis that the conserved staphylococcal and streptococcal superantigen fold has been used by the SET family to produce a group of proteins, each with differing functions.

One common feature of superantigen exposure is vomiting and diarrhea. Little is known about how superantigen structure relates to emesis and diarrhea, and how these activities are associated to superantigenicity. Recent work indicates that the emetic properties of SAgs may not be completely correlated with their superantigenicity (Harris and Betley, 1995). His225 of SEA has been demonstrated to be important for both superantigenic and emetic activity, while His61 appears to be important only for emetic activity (Hoffman *et al*., 1996), and further areas in the N-terminal region of SEA that are important for both emetic and superantigenic function have also been identified (Harris and Betley, 1995). Carboxymethylation of histidine residues in SEB was found to abrogate emetic activity, but still induces peripheral blood cell proliferation in monkeys (Alber *et al*., 1990). This is consistent with the hypothesis that the two activities are separable in SEs. In support of this, intravenous administration of a C-terminal fragment of SEC1 was found to induce diarrhea but not emesis in primates (Spero *et al*., 1978). One of the main regions of these toxins thought to be responsible for emesis is the disulphide bond and loop (Hovde *et al*., 1994). Although the disulphide bond itself is not an absolute requirement for emetic activity, the conformation within or adjacent to the loop is important for emesis. Further work is required in order to assess the contribution of certain amino acids to the various biological activities shown by SAgs in the context of the diseases they cause.

## FORMATION OF THE TRIMERIC COMPLEX FOR SIGNAL TRANSDUCTION

The complex course of events at the cell surface that leads to the formation of the MHC II·SAg·TCR complex is still ambiguous. The prospect of dimeric SAgs and SAgs with multiple MHC class II binding sites being able to form complexes with more than one MHC class II molecule or TCR means that even further diversity exists in the ability of these toxins to interact with the immune system.

When considering the formation of a complex, the character of membrane-bound receptors must be taken into account (Davis *et al*., 2003). In order for a complex to form, cell membranes need to be in close proximity to each other, and the receptors must diffuse to the site of interaction. It has been shown that in order for superantigenic T cell activation to occur, less than 0.3% of the MHC class II molecules must be occupied by superantigen (Lavoie *et al*., 2001). At higher concentrations of bound toxin, the resulting T cell response is aborted by apoptosis after a few cell divisions. Thus, a low local concentration of MHC class II molecules on the cell interface is preferable for optimum superantigenicity. It is thought that the binding of a superantigen to TCR induces clustering of the TCRs on the cell surface and the acquirement of the intracellular components required to transduce a signal (Germain, 1997). This is thought to occur in a manner that mimics peptide antigens with regard to receptor clustering (Woodland *et al*., 1997), either through direct clustering events as proposed by the TCR oligomerization model or by the binding of superantigen homodimers to multiple MHC class II molecules, which in turn would promote T cell clustering (Tiedemann *et al*., 1995). Molecular modeling suggests that signal transduction stimulated by SEA through large-scale assembly is limited to four or five TCR·(DR1β·SEA·DR1α) tetramers and requires the dimerization of MHC class II molecules. While TCRs would be clustered together in this model, TCR dimerization is thought unlikely (Cuff *et al*., 2003). SEA is not unique in its ability to form zinc-mediated dimers (Tiedemann *et al*., 1995). SED (Sundstrom *et al*., 1996) and SEC2 (Papageorgiou *et al*., 2004) can form zinc-dependent homodimers, while SpeA1 forms a non–zinc mediated disulphide-linked dimer (Baker *et al*., 2004b), and a SpeC dimer is also formed in the absence of zinc (Roussel *et al*., 1997). MAM has also been shown to be able to form zinc-dependent (Langlois *et al*., 2003) and zinc-independent dimers (Zhao *et al*., 2004); YPM is thought to be able to form zinc-independent trimers (Donadini *et al*., 2004). Clearly, SAgs have evolved slightly different ways of inducing receptor clustering. Superantigens that act as monomers and possess only a single MHC class II binding site appear to rely on the interactions of the TCR $V_\alpha$ with MHC class II-β1, which increases the stability of the ternary complex to within the range seen for conventional antigens. A stable MHC-superantigen-TCR complex with an extended half-life would therefore assist receptor clustering.

## ACKNOWLEDGMENTS

The superantigen research in KRA's laboratory was supported by the Medical Research Council (UK).

## REFERENCES

Abe, J., Takeda, T., Watanabe, Y., Nakao, H., Kobayashi, N., Leung, D.Y. and Kohsaka, T. (1993). Evidence for superantigen production by *Yersinia pseudotuberculosis*. *J. Immunol*. **151,** 4183–4188.

Abrahmsen, L., Dohlsten, M., Segren, S., Bjork, P., Jonsson, E. and Kalland, T. (1995). Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A. *Embo. J*. **14,** 2978–2986.

Acharya, K.R., Passalacqua, E.F., Jones, E.Y., Harlos, K., Stuart, D.I., Brehm, R.D. and Tranter, H.S. (1994). Structural basis of superantigen action inferred from crystal structure of toxic-shock syndrome toxin-1. *Nature* **367,** 94–97.

Alber, G., Hammer, D.K. and Fleischer, B. (1990). Relationship between enterotoxic- and T lymphocyte-stimulating activity of staphylococcal enterotoxin B. *J. Immunol*. **144,** 4501–4506.

Al-Shangiti, A.M., Naylor, C.E., Nair, S.P., Briggs, D.C., Henderson, B. and Chain, B.M. (2004) Structural relationships and cellular tropism of staphylococcal superantigen-like proteins. *Infect. Immun*. **72,** 4261–4270.

Arcus, V.L., Langley, R., Proft, T., Fraser, J.D. and Baker, E.N. (2002). The three-dimensional structure of a superantigen-like protein, SET3, from a pathogenicity island of the *Staphylococcus aureus* genome. *J. Biol. Chem*. **277,** 32274–32281.

Avena, R.M. and Bergdoll, M.S. (1967). Purification and some physicochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. *Biochemistry* **6,** 1474–1480.

Baker, H.M., Proft, T., Webb, P.D., Arcus, V.L., Fraser, J.D. and Baker, E.N. (2004a). Crystallographic and mutational data show that the streptococcal pyrogenic exotoxin J can use a common binding surface for T cell receptor binding and dimerization. *J. Biol. Chem*. **279,** 38571–38576.

Baker, M.D. and Acharya, K.R. (2003) Superantigens. Structure, function, and diversity. *Methods Mol. Biol*. **214,** 1–31.

Baker, M.D. and Acharya, K.R. (2004). Superantigens: structure-function relationships. *Int. J. Med. Microbiol*. **293,** 529–537.

Baker, M.D., Gendlina, I., Collins, C.M. and Acharya, K.R. (2004b). Crystal structure of a dimeric form of streptococcal pyrogenic exotoxin A (SpeA1). *Protein Sci*. **13,** 2285–2290.

Bernatchez, C., Al-Daccak, R., Mayer, P.E., Mehindate, K., Rink, L., Mecheri, S. and Mourad, W. (1997). Functional analysis of Mycoplasma arthritidis-derived mitogen interactions with class II molecules. *Infect. Immun*. **65,** 2000–2005.

Brehm, R.D., Tranter, H.S., Hambleton, P. and Melling, J. (1990). Large-scale purification of staphylococcal enterotoxins A, B, and C2 by dye ligand affinity chromatography. *Appl. Environ. Microbiol*. **56,** 1067–1072.

Chu, F.S., Thadhani, K., Schantz, E.J. and Bergdoll, M.S. (1966). Purification and characterization of staphylococcal enterotoxin A. *Biochemistry* **5,** 3281–3289.

Cole, B.C. (1991). The immunobiology of *Mycoplasma arthritidis* and its superantigen MAM. *Curr. Top. Microbiol. Immunol*. **174,** 107–119.

Cole, B.C., Knudtson, K.L., Oliphant, A., Sawitzke, A.D., Pole, A., Manohar, M., Benson, L.S., Ahmed, E. and Atkin, C.L. (1996). The sequence of the *Mycoplasma arthritidis* superantigen, MAM: identification of functional domains and comparison with microbial superantigens and plant lectin mitogens. *J. Exp. Med*. **183,** 1105–1110.

Cuff, L., Ulrich, R.G. and Olson, M.A. (2003). Prediction of the multimeric assembly of staphylococcal enterotoxin A with cell-surface protein receptors. *J. Mol. Graph. Model*. **21,** 473–486.

Davis, S.J., Ikemizu, S., Evans, E.J., Fugger, L., Bakker, T.R. and van der Merwe, P.A. (2003). The nature of molecular recognition by T cells. *Nat. Immunol*. **4,** 217–224.

Deresiewicz, R.L., Woo, J., Chan, M., Finberg, R.W. and Kasper, D.L. (1994). Mutations affecting the activity of toxic shock syndrome toxin-1. *Biochemistry* **33,** 12844–12851.

Deringer, J.R., Ely, R.J., Stauffacher, C.V. and Bohach, G.A. (1996). Subtype-specific interactions of type C staphylococcal enterotoxins with the *T*-cell receptor. *Mol. Microbiol*. **22,** 523–534.

Donadini, R., Liew, C.W., Kwan, A.H., Mackay, J.P. and Fields, B.A. (2004). Crystal and solution structures of a superantigen from *Yersinia pseudotuberculosis* reveal a jelly-roll fold. *Structure (Camb)* **12,** 145–156.

Ende, I.A., Terplan, G., Kickhofen, B. and Hammer, D.K. (1983). Chromatofocusing: a new method for purification of staphylococcal enterotoxins B and C1. *Appl. Environ. Microbiol*. **46,** 1323–1330.

Etongue-Mayer, P., Langlois, M.A., Ouellette, M., Li, H., Younes, S., Al-Daccak, R. and Mourad, W. (2002). Involvement of zinc in the binding of Mycoplasma arthritidis-derived mitogen to the proximity of the HLA-DR binding groove regardless of histidine 81 of the beta chain. *Eur. J. Immunol*. **32,** 50–58.

Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A. and McLaughlin, R. (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. USA* **98,** 4658–4663.

Fields, B.A., Malchiodi, E.L., Li, H., Ysern, X., Stauffacher, C.V., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (1996). Crystal structure of a *T*-cell receptor β-chain complexed with a superantigen. *Nature* **384,** 188–192.

Fraser, J.D., Urban, R.G., Strominger, J.L. and Robinson, H. (1992). Zinc regulates the function of two superantigens. *Proc. Natl. Acad. Sci. USA* **89,** 5507–5511.

Germain, R.N. (1997). T cell signaling: the importance of receptor clustering. *Curr. Biol*. **7,** R640–644.

Hakansson, M., Petersson, K., Nilsson, H., Forsberg, G., Bjork, P., Antonsson, P. and Svensson, L.A. (2000). The crystal structure of staphylococcal enterotoxin H: implications for binding properties to MHC class II and TCR molecules. *J. Mol. Biol*. **302,** 527–537.

Harris, T.O. and Betley, M.J. (1995). Biological activities of staphylococcal enterotoxin type A mutants with N-terminal substitutions. *Infect. Immun*. **63,** 2133–2140.

Hoffman, M., Tremaine, M., Mansfield, J. and Betley, M. (1996). Biochemical and mutational analysis of the histidine residues of staphylococcal enterotoxin A. *Infect. Immun*. **64,** 885–890.

Hovde, C.J., Marr, J.C., Hoffmann, M.L., Hackett, S.P., Chi, Y.I., Crum, K.K., Stevens, D.L., Stauffacher, C.V. and Bohach, G.A. (1994). Investigation of the role of the disulphide bond in the activity and structure of staphylococcal enterotoxin C1. *Mol. Microbiol*. **13,** 897–909.

Hudson, K.R., Robinson, H. and Fraser, J.D. (1993). Two adjacent residues in staphylococcal enterotoxins A and E determine T cell receptor Vβ specificity. *J. Exp. Med*. **177,** 175–184.

Ito, Y., Seprenyi, G., Abe, J. and Kohsaka, T. (1999). Analysis of functional regions of YPM, a superantigen derived from Gram-negative bacteria. *Eur. J. Biochem*. **263,** 326–337.

Jardetzky, T.S., Brown, J.H., Gorga, J.C., Stern, L.J., Urban, R.G., Chi, Y.I., Stauffacher, C., Strominger, J.L. and Wiley, D.C. (1994).

Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature* **368,** 711–718.

Kappler, J., Kotzin, B., Herron, L., Gelfand, E.W., Bigler, R.D., Boylston, A., Carrel, S., Posnett, D.N., Choi, Y. and Marrack, P. (1989). V β-specific stimulation of human T cells by staphylococcal toxins. *Science* **244,** 811–813.

Kappler, J.W., Herman, A., Clements, J. and Marrack, P. (1992). Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. *J. Exp. Med*. **175,** 387–396.

Kim, J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1994). Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. *Science* **266,** 1870–1874.

Kline, J.B. and Collins, C.M. (1997). Analysis of the interaction between the bacterial superantigen streptococcal pyrogenic exotoxin A (SpeA) and the human T-cell receptor. *Mol. Microbiol*. **24,** 191–202.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui, L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumaru, H., Maruyama, A., Murakami, H., Hosoyama, A., Mizutani-Ui, Y., Takahashi, N.K., Sawano, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamashita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H. and Hiramatsu, K. (2001). Whole genome sequencing of meticillin-resistant *Staphylococcus aureus. Lancet* **357,** 1225–1240.

Langlois, M.A., El Fakhry, Y. and Mourad, W. (2003). Zinc-binding sites in the N terminus of Mycoplasma arthritidis-derived mitogen permit the dimer formation required for high-affinity binding to HLA-DR and for T cell activation. *J. Biol. Chem*. **278,** 22309–22315.

Lavoie, P.M., McGrath, H., Shoukry, N.H., Cazenave, P.A., Sekaly, R.P. and Thibodeau, J. (2001). Quantitative relationship between MHC class II-superantigen complexes and the balance of T cell activation versus death. *J. Immunol*. **166,** 7229–7237.

Leder, L., Llera, A., Lavoie, P.M., Lebedeva, M.I., Li, H., Sekaly, R.P., Bohach, G.A., Gahr, P.J., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (1998). A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor β chain and major histocompatibility complex class II. *J. Exp. Med*. **187,** 823–833.

Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (1998a). Three-dimensional structure of the complex between a T cell receptor β chain and the superantigen staphylococcal enterotoxin B. *Immunity* **9,** 807–816.

Li, H., Llera, A. and Mariuzza, R.A. (1998b). Structure-function studies of T cell receptor superantigen interactions. *Immunol. Rev*. **163,** 177–186.

Li, Y., Li, H., Dimasi, N., McCormick, J.K., Martin, R., Schuck, P., Schlievert, P.M. and Mariuzza, R.A. (2001). Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity* **14,** 93–104.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. and Mariuzza, R. (2004). Standard nomenclature for the superantigens expressed by Staphylococcus: International Nomenclature Committee for Staphylococcal Superantigens. *J. Infect. Dis*. **189,** 2334–2336.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248,** 705–711.

Munson, S.H., Tremaine, M.T., Betley, M.J. and Welch, R.A. (1998). Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus. Infect. Immun*. **66,** 3337–3348.

Omoe, K., Hu, D.L., Takahashi-Omoe, H., Nakane, A. and Shinagawa, K. (2003). Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect. Immun*. **71,** 6088–6094.

Orwin, P.M., Leung, D.Y., Tripp, T.J., Bohach, G.A., Earhart, C.A., Ohlendorf, D.H. and Schlievert, P.M. (2002). Characterization of a novel staphylococcal enterotoxin-like superantigen, a member of the group V subfamily of pyrogenic toxins. *Biochemistry* **41,** 14033–14040.

Papageorgiou, A.C. and Acharya, K.R. (2000). Microbial superantigens: from structure to function. *Trends Microbiol*. **8,** 369–375.

Papageorgiou, A.C., Baker, M.D., McLeod, J.D., Goda, S.K., Manzotti, C.N., Sansom, D.M., Tranter, H.S. and Acharya, K.R. (2004). Identification of a secondary zinc-binding site in staphylococcal enterotoxin C2. Implications for superantigen recognition. *J. Biol. Chem*. **279,** 1297–1303.

Papageorgiou, A.C., Collins, C.M., Gutman, D.M., Kline, J.B., O'Brien, S.M., Tranter, H.S. and Acharya, K.R. (1999). Structural basis for the recognition of superantigen streptococcal pyrogenic exotoxin A (SpeA1) by MHC class II molecules and T cell receptors. *EMBO J*. **18,** 9–21.

Petersson, K., Hakansson, M., Nilsson, H., Forsberg, G., Svensson, L.A., Liljas, A. and Walse, B. (2001). Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *EMBO J*. **20,** 3306–3312.

Petersson, K., Pettersson, H., Skartved, N.J., Walse, B. and Forsberg, G. (2003). Staphylococcal enterotoxin H induces V α-specific expansion of T cells. *J. Immunol*. **170,** 4148–4154.

Proft, T., Moffatt, S.L., Berkahn, C.J. and Fraser, J.D. (1999). Identification and characterization of novel superantigens from Streptococcus pyogenes. *J. Exp. Med*. **189,** 89–102.

Reynolds, D., Tranter, H.S., Sage, R. and Hambleton, P. (1988). Novel method for purification of staphylococcal enterotoxin A. *Appl. Environ. Microbiol*. **54,** 1761–1765.

Robern, H., Stavric, S. and Dickie, N. (1975). The application of QAE-Sephadex for the purification of two staphylococcal enterotoxins. I. Purification of enterotoxin C2. *Biochim. Biophys. Acta*. **393,** 148–158.

Roussel, A., Anderson, B.F., Baker, H.M., Fraser, J.D. and Baker, E.N. (1997). Crystal structure of the streptococcal superantigen SPE-C: dimerization and zinc binding suggest a novel mode of interaction with MHC class II molecules. *Nat. Struct. Biol*. **4,** 635–643.

Schlievert, P.M., Jablonski, L.M., Roggiani, M., Sadler, I., Callantine, S., Mitchell, D.T., Ohlendorf, D.H. and Bohach, G.A. (2000). Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals. *Infect. Immun*. **68,** 3630–3634.

Spero, L. and Morlock, B.A. (1978). Biological activities of the peptides of staphylococcal enterotoxin C formed by limited tryptic hydrolysis. *J. Biol. Chem* **253,** 8787–8791.

Stevens, K.R., Van, M., Lamphear, J.G. and Rich, R.R. (1996). Altered orientation of streptococcal superantigen (SSA) on HLA-DR1 allows unconventional regions to contribute to SSA Vβ specificity. *J. Immunol*. **157,** 4970–4978.

Sundberg, E. and Jardetzky, T.S. (1999). Structural basis for HLA-DQ binding by the streptococcal superantigen SSA. *Nat. Struct. Biol*. **6,** 123–129.

Sundberg, E.J., Li, H., Llera, A.S., McCormick, J.K., Tormo, J., Schlievert, P.M., Karjalainen, K. and Mariuzza, R.A. (2002). Structures of two streptococcal superantigens bound to TCR β chains reveal diversity in the architecture of T cell signaling complexes. *Structure* (Camb) **10,** 687–699.

Sundstrom, M., Abrahmsen, L., Antonsson, P., Mehindate, K., Mourad, W. and Dohlsten, M. (1996). The crystal structure of Staphylococcal enterotoxin type D reveals $Zn^{2+}$-mediated homodimerization. *EMBO J*. **15,** 6832–6840.

Swaminathan, S., Furey, W., Pletcher, J. and Sax, M. (1992). Crystal structure of Staphylococcal enterotoxin B, a superantigen. *Nature* **359,** 801–806.

Swaminathan, S., Furey, W., Pletcher, J. and Sax, M. (1995). Residues defining Vβ specificity in staphylococcal enterotoxins. *Nat. Struct. Biol*. **2,** 680–686.

Thomas, P., Webb, P.D., Handley, V. and Fraser, J.D. (2004). Identification & characterization of the two novel streptococcal pyrogenic exotoxins, SPE-L and SPE-M. *Indian J. Med. Res*. **119** *Suppl***,** 37–43.

Tiedemann, R.E., Urban, R.J., Strominger, J.L. and Fraser, J.D. (1995). Isolation of HLA-DR1 (staphylococcal enterotoxin A) 2 trimers in solution. *Proc. Natl. Acad. Sci. USA* **92,** 12156–12159.

Uchiyama, T., Miyoshi-Akiyama, T., Kato, H., Fujimaki, W., Imanishi, K. and Yan, X.J. (1993). Superantigenic properties of a novel mitogenic substance produced by Yersinia pseudotuberculosis isolated from patients manifesting acute and systemic symptoms. *J. Immunol*. **151,** 4407–4413.

Wen, R., Broussard, D.R., Surman, S., Hogg, T.L., Blackman, M.A. and Woodland, D.L. (1997). Carboxy-terminal residues of major histocompatibility complex class II-associated peptides control the presentation of the bacterial superantigen toxic shock syndrome toxin-1 to T cells. *Eur. J. Immunol*. **27,** 772–781.

Williams, R.J., Ward, J.M., Henderson, B., Poole, S., O'Hara, B.P., Wilson, M. and Nair, S.P. (2000). Identification of a novel gene cluster encoding staphylococcal exotoxin-like proteins: characterization of the prototypic gene and its protein product, SET1. *Infect. Immun*. **68,** 4407–4415.

Woodland, D.L., Wen, R. and Blackman, M.A. (1997). Why do superantigens care about peptides? *Immunol. Today* **18,** 18–22.

Zhao, Y., Li, Z., Drozd, S.J., Guo, Y., Mourad, W. and Li, H. (2004). Crystal structure of Mycoplasma arthritidis mitogen complexed with HLA-DR1 reveals a novel superantigen fold and a dimerized superantigen-MHC complex. *Structure* (Camb) **12,** 277–288.

SECTION V

# CLINICAL, IMMUNOLOGICAL ASPECTS AND APPLICATIONS OF BACTERIAL PROTEIN TOXINS IN CELL BIOLOGY AND THERAPY

CHAPTER

# 54

# Induction and modulation of inflammatory networks by bacterial protein toxins

*Steffen Backert, Wolfgang König, Ralf Arnold, and Brigitte König*

## INTRODUCTION

The field of cellular microbiology and in this regard the knowledge of the precise function of bacterial protein toxins has broadened our understanding in microbial pathogenicity, cellular biology, and immunology. It is well known that toxins by themselves may activate, and/or down-regulate not only individual cellular functions but also the complex network in host defense. Recently, it became obvious that an understanding of infectious pathobiology also needs to consider the combined action of bacterial toxins in conjunction with the whole microorganism, as well as its soluble exoproducts in a complete system. This may result in altered cellular responses compared to the individual toxin on its own. In addition, not only single cells but the cellular network of epithelial and endothelial cells, leukocytes, lymphocytes, fibroblasts as well as smooth muscle cells are activated by microbial toxins alone, as well as by microbial exoproducts in combination with defined toxins (Beutler, 2004; Boyle and Finlay, 2003; Brandtzaeg and Pabst, 2004; Brom *et al*., 1992; Burns *et al*., 2003; Janeway, 1989; Kaufmann *et al*., 2004; Maus *et al*., 2004). Obviously, one has to analyze primarily the action of a particular toxin with a defined cell to understand its mode of action. For our knowledge on the pathobiology of infectious diseases, the mechanisms of host defenses and potential anti-infective strategies, one has to consider a highly complex scenario of different cells and inflammatory mediators.

The past years have led to a novel understanding with respect to the recognition of microorganisms, and toxins, as well as soluble exoproducts, by delineating the importance of the innate immune system and also by the recent progress in understanding how microbial signals are received and transmitted from the cell surface via various steps of the signal transduction cascade leading to the induction of cellular responses (Akira *et al*., 2001; Baggiolini, 2001; Barbieri *et al*., 2002; Beutler, 2004; Boquet, 2003; Chen and Greene, 2004; Cook *et al*., 2004; Finlay and Falkow, 1997; Galan and Collmer, 1999; Henderson *et al*., 1996; Hornef *et al*., 2002; Kotwal, 1997; Medzhitov and Janeway, 2000; Schiavo and van der Goot, 2001; Zychlinsky and Sansonetti, 1997). In this chapter, we will focus on individual pathogenicitiy factors, but also on defined and up to now unresolved disease processes. The complexity of host defense will be described with emphasis on the mucosal system, as well as inflammatory cells and mediators.

### Microbial recognition—role of epithelial and dendritic cells

The mucosal immune system has the complex task of responding to a vast number of signals presented by the myriad of ingested and inhaled antigens. Under normal physiological conditions, immunological tolerance is induced to food, airborne antigens, and commensal bacteria, whereas potent effector immune responses are generated only to dangerous pathogenic

microorganisms (Kelsall and Rescigno, 2004; Svanborg *et al*., 1999). In contrast, in pathological conditions such as allergy or inflammatory bowel disease, an abnormal immune response to harmless antigens results in damaging inflammation (Hadjivassiliou *et al*., 2004). How the discrimination between dangerous and innocuous antigens is achieved at mucosal surfaces is not yet fully clear and is presently being investigated. A principal function has been raised for epithelial as well as for dendritic cells in regulating the induction of mucosal immune responses. In particular, data have emerged that mucosal dendritic cells have unique functions that are not shared by dendritic cells from other tissues, suggesting that the tissue microenvironment can influence the phenotype and functional responses of dendritic cells (Kagnoff and Eckmann, 1997; Liston and McColl, 2003; Mackay, 2001; Muzio *et al*., 2000; Re and Strominger, 2001; Romani *et al*., 1997; Shortman and Wu, 2004; Silverman and Fitzgerald, 2004; Visintin *et al*., 2001; Vogelmann *et al*., 2004).

Epithelial cells are actively involved in determining how mucosal dendritic cells take up antigens. In organized mucosal tissues, such as Peyer's patches and colonic follicles, epithelial cell-derived M cells transport antigens directly from the lumen to underlying dendritic cells. Clearly, a distinction of the function of dendritic cells is obtained by the differentiation of these cells under the influence of various cytokines and a complex cellular network. Obviously, epithelial cells in this regard are not passive barriers but by virtue of chemotactic factors generate an epithelial-endothelial link allowing the transmigration of neutrophils from the blood vessel into the tissue. Neutrophils, once activated, combat pathogenic microorganisms. The network of inflammatory mediators, as well as the cross-talk of various cells under the influence of defined microbial antigens and toxins, allows the priming of regulatory T cells (Treg) with the generation of Th1 and Th2 immune responses.

Recent data on the epitope specificity of microbial antigens have led to novel ideas as to the mechanisms of intestinal colonization, microbial invasion, as well as microbially induced inflammation (Arnold *et al*., 1993; Brandtzaeg and Pabst, 2004; Cossart and Sansonetti, 2004; Dalton *et al*., 2004; Frandji *et al*., 1996; Galli, 1997; Mantovani *et al*., 1997; Monack *et al*., 2004).

## Bacterial protein toxins, inflammatory responses, and signals

Protein toxin release by bacteria can activate and paralyze host defense mechanisms in many ways, which ultimately also affects inflammatory responses (Brom and König, 1992; Eriksson *et al*., 2000; Hensler *et al*., 1994; Hensler *et al*., 1991; Holst, 2003; König and König, 1991; König and König, 1993; König, B. *et al*., 1997). The cell biological action of several protein toxins relevant to this chapter will be described. With the exception of pore-forming toxins, most bacterial protein toxins have an enzymatic activity towards specific cytoplasmic targets (Tomita and Kamio, 1997). This implies that the toxin must cross the bilayer and penetrate the target cell (Burns *et al*., 2003). Toxins often undergo endocytosis followed by transport to specific intracellular organelles before they translocate into the cytosol. In many cases, this coincides with the activation of nuclear responses, such as transcription factor NF-κB or AP-1, involved in regulating the expression of inflammatory mediators and programmed cell death (apoptosis) modulators (Chen and Greene, 2004; De Haan and Hirst, 2004; Dragneva *et al*., 2001; Hollenbach *et al*., 2004; Kayal *et al*., 2002). For example, infection with Stx-positive enterohemorrhagic *E. coli* (EHEC), a gastrointestinal pathogen that causes acute intestinal inflammation and diarrhea, activated p38 and Erk (extracellular-regulated kinase), two typical mitogen-activated protein kinases (MAPK), and the nuclear translocation of NF-κB leading to IL-8 secretion. In addition, cytokine expression arrays were used to determine the effect of recombinant Stx1 on various chemokine genes at the mRNA level in intestinal epithelial cells, including IL-8, GRO-α, GRO-β, GRO-γ, and ENA-78 (Berin *et al*., 2002; Hauf and Chakraborty, 2003). *Staphylococcus aureus* α-toxin is a pore-forming exotoxin that has been implicated as a significant virulence factor in human staphylococcal diseases. Purified α-toxin provoked the rapid-onset phosphatidylinositol (PtdIns) hydrolysis, as well as the liberation of nitric oxide (NO) and the prostanoids $PGE_2$, $PGI_2$, and thromboxane $A_2$ in primary epithelial cells. In addition, α-toxin led to a sustained up-regulation of pro-inflammatory IL-8 mRNA expression and chemokine secretion. α-toxin pore-associated transmembrane ion flux and phosphatidylinositol hydrolysis-related signaling with downstream activation of protein kinase C (PKC) and nuclear translocation of NF-κB are suggested to represent important underlying mechanisms. Other protein toxins are specialized to enter host cells via specific pathways and bind to eukaryotic receptor molecules. This is very important because these toxins exhibit a remarkable cell type specificity.

For example, some toxins enter host cells through clathrin-coated vesicles, such as *Pseudomonas* exotoxin A or diphteria toxin, while others, such as heat-labile enterotoxin from *E. coli* or cholera toxin, enter through the putative caveolar pathway.

Toxins may mediate their biological effects via several mechanisms. Microbial toxins, as well as other

bacterial exoproducts, modify different classes of G proteins by ADP-ribosylation (Bishop and Hall, 2000; Black and Bliska, 2000; Boquet, 2003; Brom, J. *et al*., 1993; Chimini and Chavrier, 2000; Duesbery *et al*., 1998; Falnes and Sandvig, 2000; Gao and Kwaik, 2000; Novick, 2000; Wiegers *et al*., 1991). In this regard, cholera toxin and *E. coli* enterotoxin activate the α-subunit of heterotrimeric $G_s$ proteins downstream to stimulatory G-protein-coupled receptors ($GPCR_s$), leading to the constitutive activation of adenylyl cyclase and rapid elevation of cellular levels of cyclic AMP (cAMP). Pertussis toxin instead inactivates the α-subunit of G proteins coupled to inhibitory GPCR ($GPCR_i$). This modification causes the silencing of the inhibitory input and induces the indirect activation of downstream effectors. The mechanisms of the vacuolating cytotoxin VacA of the gastric pathogen *Helicobacter pylori*, which binds to a cellular receptor called Git1 (G protein-coupled receptor kinase-interactor 1) via interaction with the protein tyrosine phosphatase type Z (PTP-ζ) in gastric epihelial cells, has been outlined recently .

Another recurring target of bacterial protein toxins is eukaryotic cell adhesion molecules (CAMs) (Kerr, 1999). They are involved in many processes, including recognition by circulating phagocytes at the site of inflammation, transmigration through the endothelial barrier, diapedesis through the basement membrane and extracellular matrix, as well as the release of effector mechanisms at the infected site. CAMs are involved in the pathogenetic effects of the RTX toxins of *Pasteurella haemolytica* and the superantigen exotoxins of *Staphylococcus aureus* and *Streptococcus pyogenes*. The epithelial apical-junctional complex is another key regulator of cellular functions. Many pathogens exploit intestinal permeability through the modulation of the cell cytoskeleton and intercellular tight junctions to survive, proliferate, and sometimes persist within a host. In addition, another important mode of entry, bypassing the intestinal epithelial barrier, is used by *Salmonella* and *Shigella* and occurs through a specialized cell type, the M cell (Fasano and Nataro, 2004; Finlay and Falkow, 1997).

A specific class of bacterial toxins catalyzes the covalent modification of actin or Rho GTPases, which are involved in several responses, such as the control of the host actin cytoskeleton and bacterial invasion. It is important to point out that the control of the actin cytoskeleton by Rho GTPases is also directly and indirectly linked to the activation of several nuclear transcription factors, such as NF-κB, c-Jun, or Elk-1, involved in regulating the expression of inflammatory mediators, defenses, and apoptosis modulators (Bishop and Hall, 2000; Black and Bliska, 2000; Hensler *et al*., 1994b; Just *et al*., 1995; MacMicking, 2004; Marinissen and Gutkind, 2001). Rho GTPase regulation of inflammatory cytokines and cell death modulators might also reflect its participation in the host-cell defense. Consistent with this interpretation is that Rac positively regulates NADPH oxidase, thus directly linking Rho GTPases to pathogen attack. The NADPH oxidase complex is composed of several subunits in the plasma membrane (cytochrome $b_{558}$ subunits $p22^{phox}$ and $gp91^{phox}$) and a number of activity-regulating proteins in the cytoplasm ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$). This NADPH oxidase complex assembles to generate superoxide ($O_2^-$) in phagocytes, which may then react with chloride ions to generate hypochlorous acid (HOCl). In this way, neutrophils form a highly toxic environment within the phagosomes and in the cell surroundings to kill a broad range of microbes (Quinn and Gauss, 2004). *Pseudomonas* ExoS/T block bacterial host cell entry and, by inhibiting activation of NF-κB, interfere with innate immunity mediators but facilitate apoptosis through a lack of production of anti-apoptotic factors. Activators of Rho, Rac, and Cdc42 have an opposite effect, inducing phagocytosis, blocking apoptosis, and activating the inflammatory response. Consequently, activation or down-regulation of this GTPase signaling by bacterial protein toxins has important impact on inflammatory responses (Allen, 2003; Berger *et al*., 2002; Brom, J. *et al*., 1993; Raveh *et al*., 1998).

Toxins can also mediate their biological effects by interfering with kinases and via generation of prostanoids directly and indirectly with nuclear hormone receptors, such as peroxisome proliferators-activated receptors (PPAR). These receptors function as an important regulator of cell differentiation, proliferation, and apoptosis in various cell types, i. e., stromal cells (adipocyte, macrophage, endothelium, and smooth muscle, as well as parenchymal epithelial cells).

PPAR-γ is also a key mediator in cellular signaling pathways, such as Akt, MAPK, TGF-β, and NF-κB. Quite recently, it was shown that PPAR-γ agonists have anti-inflammatory effects and reduce neutrophil influxes in various compartments of the mucosal system, e. g., the lung (Arnold and König, 2004b; Asada *et al*., 2004; Murphy and Holder, 2000, Riches, 2004). Interestingly, recent data demonstrated that CD36, a scavenger receptor, is up-regulated by PPAR-γ on mononcytes and macrophages. PPAR-γ modulates CD36 gene expression through direct interaction with the proximal promoter via a specific response element. The primary function of CD36 has been shown to be associated with the clearance of apoptotic cells arising during development, normal homeostasis, and

inflammation. At the inflammatory sites, CD36 functions as a receptor for apoptotic neutrophils, which mediates their phagocytosis by macrophages. During early inflammatory processes, neutrophils are recruited from the circulation in response to a series of coordinated signals. In the resolving phase, the accumulated neutrophils undergo apoptosis and are subsequently phagocytosed by macrophages. This removal of apoptotic neutrophils by macrophage phagocytosis is a critical process for the resolution of inflammation. In the lung, it is thus hypothesized that the activation of PPAR-γ enhances CD36 expression by alveolar macrophages, which in turn augments their phagocytic capacity for apoptotic neutrophils.

This regulatory process is impaired in cystic fibrosis. A recent study demonstrated an increased local production of 15d-$PGJ_2$ in acute lung inflammation (Riches, 2004). The exudate levels of prostaglandins, such as $PGE_2$, $PGD_2$, and 15d-$PGJ_2$, were measured. It was shown that $PGD_2$ and 15d-$PGJ_2$ increased at both initial and, to a greater extent, later phases of lung inflammation, while $PGE_2$ was produced only at the initial phase. These data suggested that the profiles of prostaglandins produced during inflammation may change from a $PGE_2$-dominant profile to $PGD_2$- and 15d-$PGJ_2$-dominant pattern during the resolution of inflammation. Based on these findings, locally generated 15d-$PGJ_2$ during the late phase of lung inflammation could activate PPAR-γ in alveolar macrophages, which in turn reduces their cytokine production and increases their CD36 expression together with an enhancement of their phagocytic capacity. A dysregulation of these events is obtained during bacterial and viral infection. Obviously, bacterial toxins can induce an increase in free cytosolic $Ca^{2+}$ in targeted host cells, required for the toxin-mediated effects. Toxin-induced $Ca^{2+}$ responses may result in NF-κB-dependent activation of gene expression, as reported for the induction of IL-6, IL-8, or mucin. Thus, $Ca^{2+}$ acts as an important messenger used by toxins to regulate intracellular and intercellular signaling pathways. Such signals can induce or amplify cellular responses towards pathogens, and are likely to play a crucial role in mounting and modulating the inflammatory response (TranVan Nhieu *et al*., 2004).

## The role of chemokines and their receptors

Microbial toxin recognition and induction of inflammation is mediated by chemokines or chemotatic cytokines, which have chemoattractant properties for inflammatory cells (Bergmann *et al*., 1989; Hensler *et al*., 1994; König and König, 1993; König, B. *et al*., 1996a; König, B. *et al*., 1994a; König, W. *et al*., 1990). Chemokines are commonly divided into four structural families, based on the spacing of highly conserved cysteine residues (CXC, CC, C, and $CX_3C$). Whereas the CXC chemokines are involved in neutrophil recruitment and activation, the CC chemokines act towards various lymphocyte subsets, monocytes, eosinophils, and basophils. About 50 human chemokines and nearly 20 receptors have been identified and characterized since the discovery of IL-8 (Baggiolini, 2001; Baggiolini and Clark-Lewis, 1992; Kunkel *et al*., 199; Loetscher and Clark-Lewis, 2001; Mackay, 2001; Mahalingam and Karupiah, 1999; Stevenson *et al*., 2004; Tsai *et al*., 2000). Cytokines and chemokines regulate immune function by controlling growth, proliferation, differentiation, and migration of leukocytes. Many cytokines act via pathways such as the Jak/STAT and MAPK pathways. Chemokines are small structurally-related cytokines that induce migration of various immune cells via interactions with the seven transmembrane GPCRs. They can activate protein kinases such as ERK, JNK, and AKT to induce cell growth. Other chemoattractants, like fMLP, a formylated tripeptide released by bacteria at the site of infection, also signal via GPCRs and thereby induce migration of phagocytic cells such as neutrophils, macrophages, and monocytes. Neutrophil chemoattractants like IL-8 and fMLP regulate cell migration and extravasation, and thus attract cells toward inflammatory sites. Both fMLP and IL-8 can also cause the release of granular enzymes, oxygen burst, and stimulate the adherence of neutrophils to endothelial cells via adhesion molecules such as Mac-1.

IL-8 is the prototype of CXC chemokines and the best-studied member of this group. Increased IL-8 levels have been observed in many bacterial infections and obtained from various sites of infection, e. g., sputa from CF, mucin of gastrointestinal diseases (Becker *et al*., 2004; Dai *et al*., 1994). Microbial infection and activation of inflammatory cells may also increase gene expression of a specific receptor for IL-8, the CXC-R1 (CXC receptor 1). IL-8 in addition to its chemotactic and granulocytic activating and secretory properties has been implicated to reduce the potency of IFN-α *in vivo*, thus aggravating viral infections. Thus, bacterial exoproducts and toxins (e.g., leukocidins) may also contribute via IL-8 to viral infection. IL-8 also stimulates endothelial cells by activation of CXCR1 and CXCR2 and thus facilitates cell polarization and migration in response to chemokines. The capacity of the different microbes, their exoproducts, and toxins to trigger IL-8 release is a subject of significant variability (Deng *et al*., 2004; Ji *et al*., 1997; Köller *et al*., 1993). Depending on the presence of pro-inflammatory cytokines, various microorganisms by virtue of distinct pathogenicity

factors are able to selectively recruit inflammatory cells (Gröner *et al*., 1992; Howard *et al*., 2001; König, W. *et al*., 1992). For each of these agents, receptors are expressed on the granulocyte, which signal through the seven-span superfamily of integral membrane proteins with seven transmembrane domains, such as CXC-R1. The relationship between chemokines and chemokine receptors is highly promiscous, with most receptors binding many chemokines, and most chemokines binding several receptors.

Neutrophils are equipped with a machinery to sense the site of an infection, to crawl towards the pathogen, and to ingest and kill them. After egress of neutrophils from the bone marrow to the blood circulation, the neutrophils adhere to the vascular lining and move into the infected tissues. This process involves two major adhesion molecules (E-selectin [CD62E] or P-selectin [CD62P]) and is termed diapedesis or transendothelial migration (TEM). TEM of immune cells can be investigated using the Transwell *in vitro* model system. The interaction of bacteria, and most specifically protein toxins and other virulence factors, with immune effector cells does not only induce the release of IL-8; a complex network of host reactions with the generation of both pro- and anti-inflammatory molecules is also involved. For example, chemotactic peptides are released by infecting microbes (e.g., fMLP, formyl-methionyl-leucyl-phenylalanine) and the host complement system (the anaphylatoxins, the split product of activated C3 and C5 [C3a, C5a]). Lipid mediators, such as leukotriene $B_4$ ($LTB_4$) and the platelet-activating factor (PAF), are also strong chemoattractants generated by phospholipases from membrane phospholipids during toxin-as well as bacterial-induced inflammation (Iyer *et al*., 2004; König, W. *et al*., 1990; Kotone-Miyahara *et al*., 2004; Neuber *et al*., 1991). In addition, activating receptors expressed on neutrophils and monocytes infiltrating into infected tissues (but not inflammatory lesions *per se*) have been identified as the triggering receptor expressed on myeloid cells TREM-1 and TREM-2 (triggering receptor expressed on myeloid cells). These molecules are upregulated on neutrophils by bacteria (*P. aeruginosa, S. aureus*) or their products (LTA or LPS) and probably interact with a yet unknown ligand (Bouchon *et al*., 2001).

The role of chemokines is not limited to chemotaxis alone. Chemokines also function in Th1/Th2 differentiation, T cell co-stimulation, granulocyte activation, gene transcription, mitogenesis, apoptosis, hematopoesis, angiogenesis, and cell development. In the context of defining human diseases, the dichotomy of type-1 (Th1-like) and type-2 (Th2-like) cytokines is commonly used. Quite recently, the role of diverse T regulatory cells (Treg) skewing Th1 and Th2 responses have been described. In general, type-1 cytokines favor the development of a strong cellular immune response, whereas type-2 cytokines facilitate a humoral immune response; once excessively generated, they may trigger allergic inflammatory reactions (König, W. *et al*., 1991; Leung, 1998; Maggi, 1998; Neuber and König, 1992; Smith *et al*., 2004; Tvinnereim *et al*., 2004; Zhu *et al*., 2004). The production of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-8, and IL-12, by host cells upon sensing bacterial products is crucial in the innate and adaptive immune responses to infection (Hoebe *et al*., 2004; Janeway, 1989; Silverman and Drazen, 2003; Strieter *et al*., 2003; Trinchieri, 1998). The latter cytokines play a critical role in enhancing the anti-bacterial function of professional phagocytes, recruiting additional innate immune cells to sites of infection, inducing dendritic cell maturation, and directing the resulting immune response to the invading microbes. Bacterial protein toxins, microbial exoproducts, or bacterial protein toxins with microbial exoproducts combined can actually interfere with each pathway of the innate immune system and either initiate or down-regulate inflammatory responses in the course of infection (Merrell and Falkow, 2004; Moese *et al*., 2002; Vergnolle *et al*., 2001).

## Th1-/Th2-cell responses during microbial infection—contribution of innate immunity

Several aspects shall be discussed. Whether the unique functions ascribed to mucosal dendritic cells are due to their intrinsic properties or are conferred by the local microenvironment remains an open question. However, it appears that epithelial cells may have a key function in "instructing" mucosal anti-inflammatory dendritic cells. In co-culture studies of epithelial cell monolayers, dendritic cells, and bacteria, it was demonstrated that products of epithelial cells condition dendritic cells to release IL-6 and to prime Th2 responses in an allogeneic response, even after encounter with pathogenic *Salmonella typhimurium*. These Th2 responses could help to maintain the homeostasis of the gut to encountered commensal bacteria. However, a more difficult issue is how protective Th1 responses can be initiated to intracellular pathogens such as salmonella that first encounter dendritic cells in mucosal tissues, particularly because pathogens and commensal bacteria share many if not most ligands for pathogenicity molecules (such as lipopolysaccharide and flagellin) and can stimulate dendritic cells to mature and to produce cytokines similarly *in vitro* (Singer and Sansonetti, 2004). In models of Th1-mediated inflammatory bowel disease that are similar

to Crohn's disease in humans, dendritic cells seem to have a dominant function both in the induction of Th1 cell responses as well as in driving counter-regulatory T cell responses (Bodger and Crabtree, 1998; Enarsson *et al*., 2004; Ishihara *et al*., 2004; Ye *et al*., 1997). In fact, the *Helicobacter pylori*-induced inflammation is dominated by Th1 immune responses.

By analyzing the various strategies of microbial colonization, invasion, and induction of inflammation, one is certainly surprised that the mechanisms of innate and adaptive immune recognition are essential for the outcome of the result. Indeed, innate immune mechanisms shape the adaptive immune response, and inflammatory mediators released during the adaptive immune response further focus innate immune reactions (Medzhitov and Janeway, 2000). In this regard, novel concepts fit into this scheme very well and will be discussed below. Antimicrobial proteins and peptides serve as antiinfective molecules and are released from granulocytes, monocytes, macrophages, epithelial cells, mast cells, eosinophils, and lymphocytes, as well as from non-leukocyte sources.

Initial studies as to their functional activities were focused on their microbicidal actitiy (Devine, 2003; Diamond *et al*., 2000; Galli, 1997; Kao *et al*., 2004; Kaufmann *et al*., 2004; Kimata *et al*., 1996; Labor, 2000; Levy, 2004; Mécheri and David, 1997; Vergnolle *et al*., 2001). However, with the growing appreciation of the role of acute inflammatory responses triggered by the recognition of microbial surface components, there is increasing knowledge as to their effects on the activity of microbial toxins, as well as on the Toll-like receptor (TLR) pathway.

In addition, trefoil factors (TFFs) are critically involved in responses to intestinal injury after infection, primarily by their ability to promote epithelial restitution and the rapid spreading and migration of existing cells following injury. They also interfere with the binding of microorganisms to epithelial cells and thus impair their toxin production and release (Nikolaidis *et al*., 2003). TFFs are small (7–12kD), protease-resistant proteins, composed of a characteristic three-loop structure, formed by three conserved cysteine disulfide bonds, and secreted by the gastrointestinal mucosa in a lineage-specific manner. Because of the established role of defined TFF2 in gastrointestinal repair responses, TFF2 is a novel candidate gene likely to be involved in the remodeling and repair responses associated with allergic and inflammatory lung disorders. TFFs directly interact with mucin proteins, molecules that are overproduced in asthmatic and inflammatory lung responses. A specific regulation of TFF2 by diverse allergens and antigens, as well as the Th2 cytokines IL-4 and IL-13, has been shown.

## Role of Toll-like receptors (TLRs)

How innate immunity discriminates between non-invasive bacterial pathogens and commensals is unclear. It is well established that sensing of bacteria by the innate immune system is based on the interaction between pattern-recognition receptors (PRRs) and bacterial-associated molecules, such as lipopolysaccharide (LPS), peptidoglycan, DNA containing unmethylated CpG motifs, flagellin, and lipoteichoic acid absent from self-tissues (Akira *et al*., 2001; Amorim *et al*., 2002; Arbour *et al*., 2000; Beutler, 2004; Horng *et al*., 2002; Iwasaki and Medzhitov, 2004; Park *et al*., 2004; Sabroe *et al*., 2002; Shimazu *et al*., 1999). Although the bacterial elicitors of PRRs are generally defined as pathogen-associated molecular patterns (PAMPs), this definition has to be carefully used because they are structural components of both pathogenic and harmless (or even useful) bacteria (Dunzendorfer *et al*., 2004; Hajjar *et al*., 2002; Kawei *et al*., 2004; Knodler *et al*., 2001; Takeuchi *et al*., 2000).

The largest and best-studied family of PRRs comprises the TLRs, which are membrane-associated and have an external leucine-rich repeat recognition domain and an intracellular Toll-interleukin 1 receptor signaling domain. In addition to TLRs, a second class of PRRs, consisting of the Nod1 and Nod2 cytoplasmic proteins, is essential in intracellular bacterial sensing. Obviously, these protein receptors can also detect extracellular bacteria. Like TLRs, Nod proteins have a leucine-rich repeat recognition domain linked to a signaling motif called the caspase activation and recruitment domain. TLRs and Nod1 trigger similar signaling pathways characterized by the activation of the transcription factor NF-κB, leading to the production of pro-inflammatory cytokines. In contrast, Nod2 has a regulatory function in TLR signaling, which might explain why mutations in the human gene encoding Nod2 are associated with inflammatory disorders, e. g., Crohn's disease (Cook *et al*., 2004; Giradin *et al*., 2003; Gutierrez *et al*., 2002; Haehnel *et al*., 2002; Hoshino *et al*., 1999). Nod1 and Nod2 recognize two different forms of peptidoglycan-derived motifs called muropeptides, which are normally released from bacteria during growth. Nod2 is elicited by muramyl dipeptide, a muropeptide present on peptidoglycan from all bacteria, whereas Nod1 recognizes GM-tri-DAP (*meso*-diaminopimelate-containing *N*-actylglucosamine-*N*-actylmuramic acid tripeptide), which is only present in Gram-negative bacteria and in a subset of Gram-positive bacteria (Boone *et al*., 2004; Viala *et al*., 2004).

How the epithelial cells, which are constantly exposed to all classes of bacteria and are considerably challenged, discriminate between dangerous and harmless bacteria, avoiding undesirable innate imm-

une reactions leading to chronic inflammation, is currently investigated. One potential mechanism is based on compartmentalization of PRRs (De Gregorio and Rappuoli, 2004). In this regard, TLR4, which is the sole LPS sensor, is generally associated with the plasma membrane of human myeloid immune cells such as macrophages, thus reacting to extracellular LPS derived from bacteria that have invaded internal organs or bloodstream, which are usually sterile. In contrast, in intestinal epithelial cells, which are constantly exposed to external bacteria, TLR4 expression is intracellular and therefore recognizes only internalized LPS or possibly LPS associated with bacteria that have been internalized and survived in intracellular compartments. Similarly, the cytoplasmic localization of Nod1 in intestinal epithelial cells has been linked to the selective recognition of invasive pathogenic Gram-negative bacteria. In addition to the sensing by TLRs, non TLR-mediated effector functions have also been described. Furthermore, TLR polymorphisms have to be considered, leading to inadequate innate responses (Arnold *et al*., 2004; Beutler, 2004; Lazarus *et al*., 2004). Clearly, our view as to the induction of inflammatory mediators, chemokines, their receptors, the skewing of T cell responses, the maturation of dendritic and of T regulatory cells has significantly expanded and contributed in a substantial way to our modern understanding of cellular microbiology. In this regard, the research on *Helicobacter pylori*, on inflammatory mechanisms in cystic fibrosis (CF), as well as inflammatory reactions induced by bioprosthetic devices, are important examples to understand host defense mechanisms against microorganisms, toxins, and/or microbial exoproducts embedded in a biofilm. The latter components may activate the immune system, thus impairing host and antibiotic defense strategies.

In this chapter we will summarize our recent results in: (i) modulation of host cells responses by *Helicobacter pylori*, (ii) inflammatory reactions in cystic fibrosis, and (iii) the role of biopolymers and microbial exoproducts/toxins in inducing aseptic and septic inflammation.

## *HELICOBACTER PYLORI* — GENERAL VIRULENCE MECHANISMS

### Pathogenicity and virulence factors

*Helicobacter pylori* is a highly successful gastric pathogen that has been recognized as the causative agent of chronic gastric inflammation, a disease that can progress to peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, or even gastric cancer (Covacci *et al*., 1999; Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). The severity of *H. pylori*-related disease varies greatly among infected individuals and appears to be influenced by both host and bacterial factors. *H. pylori* is genetically diverse, a feature shown to contribute to the wide spectrum of clinical manifestations. Virulent isolates are characterized by the presence of disease-associated genetic components, such as the vacuolating cytotoxin (VacA) and the *cag* (cytotoxin-associated genes) pathogenicity island (*cag*PAI). The latter encodes about 30 protein components of a type IV secretion system required for the delivery of the immuno-dominant effector protein CagA and probably other proteins into the target cells. Seven of the *H. pylori cag*PAI genes are homologous to the well-characterized virulence genes *virB4, virB7, virB8, virB9, virB10, virB11*, and *virD4* from conserved type IV secretion systems of *Bordetella pertussis, Legionella pneumophila, Rickettsia prowazeki, Brucella*, and *Bartonella* species (Covacci *et al*., 1999; Backert *et al*., 2002). *In vitro* studies have shown that only *H. pylori* strains with a functional *cag*PAI induce strong cytoskeletal modifications involved in "cell scattering," as well as transcription factor NF-κB-dependent pro-inflammatory responses in gastric epithelial cells. A model showing the main virulence factors involved in persistent colonization and disease development is presented in Figure 54.1. Other known virulence determinants of *H. pylori* include: flagella-driven motility in the stomach mucus layer, local buffering of stomach acid by urease (UreA, UreB, and accesory proteins), adhesion to gastric epithelial cells mediated by several adhesins (including BabA, SabA, AlpA/B, or HopZ), and adherence of neutrophils to endothelial cells induced by the neutrophil-activating protein NapA. Other described virulence factors are the catalase KatA with a proposed function in the oxidative defense of *H. pylori* and IceA, a restriction endonuclease homologue, whose expression is induced by contact with the gastric epithelium.

We and our collaborators have established a dynamic 2-D-PAGE database with multiple subproteomes of *H. pylori* (http://www.mpiib-berlin.mpg.de/2D-PAGE), which facilitates identification of bacterial proteins important in pathogen-host interactions, as well as their subcellular localization (Backert *et al*., 2005). In a recent study using real-time quantitative (TaqMan) RT-PCR, we also determined the mRNA expression of clinical *H. pylori* isolates in response to a changing host environment, which enabled us to identify variable and strain-specific gene expression profiles for *cagA*, *ureA*, *napA*, *katA*, and *vacA* alleles in a sensitive and reproducible manner. The expression profiles of these genes varied according to the strain, and were mainly either up-regulated or unchanged upon bacterial contact with

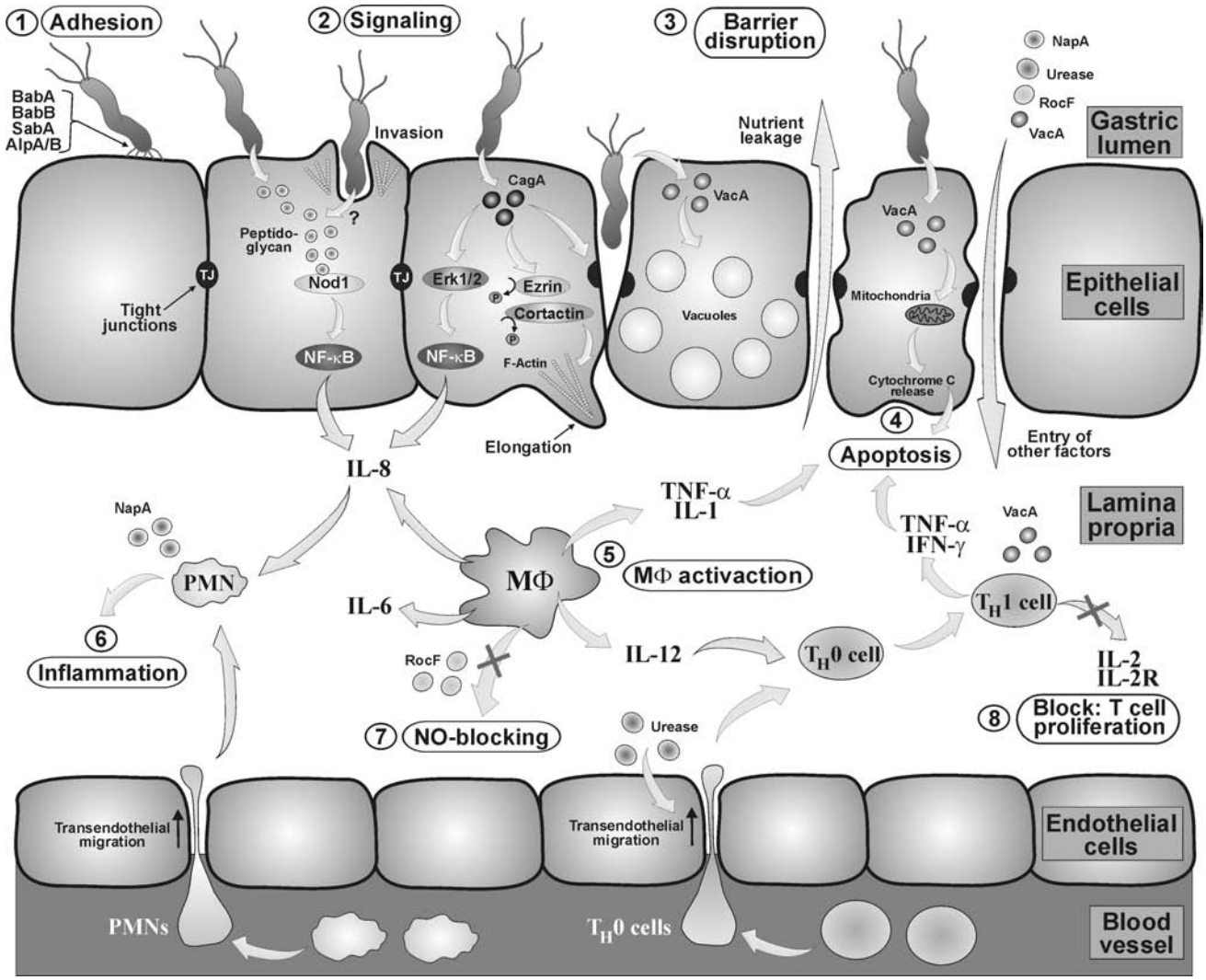



**FIGURE 54.1** Model for *Helicobacter pylori*-induced gastric damage and inflammation.
The interplay between target cell and a variety of pathogenicity factors modulates multiple host responses, leading to both chronic gastritis and persistent colonization. (1) *H. pylori* expresses several adhesins, such as BabA, SabA, and AlpA/B, that mediate binding to gastric epithelial cells. (2) After adherence, *H. pylori* can translocate effector molecules such as CagA and peptidoglycan into the host cell by a type IV secretion-dependent process. Peptidoglycan binds to the intracellular receptor Nod-1 and activates transcription factor NF-κB to induce IL-8 release (Viala *et al*., 2004). *H. pylori* is also able to occasionally enter host cells (Kwok *et al*., 2002), but it is not clear if this could contribute to the stimulation of the Nod1 pathway as known from other intracellular pathogens. Injected CagA can be phosphorylated by Src tyrosine kinases (Selbach *et al*., 2002). Phosphorylated CagA then binds to a number of host cell signaling molecules to trigger several signaling cascades (Mimuro *et al*., 2002; Selbach *et al*., 2003; Tsutsumi *et al*., 2003; Amieva *et al*., 2003). For example, CagA contributes substantially to the induction of IL-8 by activation of an Erk1/2-NF-κB-dependent pathway (Brandt *et al*., 2004). (3) Translocated CagA is also involved in the induction of overall actin cytoskeletal rearrangements and disruption of the tight junction (TJ) complex of infected host cells (Selbach *et al*., 2003; Amieva *et al*., 2003). One major effect of phosphorylated CagA is the inactivation of its own kinase (Src) by a negative feedback loop mechanism (Selbach *et al*., 2003). Inactivation of Src leads to the dephosphorylation of a number of host proteins, including cortactin and ezrin (Selbach *et al*., 2003; Selbach *et al*., 2004). These events are crucial for CagA-induced actin cytoskeletal rearrangements and host cell elongation. Disruption of the TJs by CagA and cellular effects induced by the vacuolating cytotoxin VacA contributes to the disruption of the epithelial barrier. Internalization of VacA into the cells leads to the formation of large vacuoles and gastric damage, a hallmark of the ulceration process. (4) VacA can also trigger apoptosis when recruited to the mitochondria to induce the release of cytochrome c (reviewed by Montecucco and Rappuoli, 2001; Peek and Blaser, 2002). Finally, disruption of epithelial barrier functions by *H. pylori* may cause leakage of nutrients into the gastric lumen and the ability of other pathogenicity factors to cross the epithelial layer. (5) Thereby, *H. pylori* antigens can activate macrophages (MΦ) to release several pro-inflammatory cytokines, such as IL-8, IL-6, IL-1, and IL-12. This and the aforementioned induction of IL-8 by epithelial cells, constitutes crucial determinants for the *H. pylori*-triggered inflammatory responses. (6) These cytokines can alter the secretion of mucus, because they induce changes in gastric-acid secretion and homeostasis, as well as attract other immune cells such as PMNs to infiltrate from the blood stream into the gastric mucosa. The IL-12 micro-environment induced by macrophages is particularly important for shifting the $CD4^+$ helper response into a prominent Th1 type. T cells can be recruited from the bloodstream by stimulating their transendothelial migration. Bacterial pathogenicity factors, such as the *H. pylori* urease and yet unidentified *cag*PAI components, appear to enhance the latter effect (Enarsson *et al*., 2004). The production of TNF-α, IFN-γ, and IL-1 from T cells and macrophages, respectively, supports the VacA-induced apoptosis of epithelial cells. *H. pylori* modulates the immune response by (7) inhibition of NO release from macrophages by bacterial RocF activity (Gobert *et al*., 2001) and (8) blocking T cell proliferation by VacA based on down-regulation of IL-2 and IL-2R transcription (Gebert *et al*., 2003; Bonacristiano *et al*., 2003).

AGS cells (Gieseler *et al*., 2005). In contrast, upon contact with granulocytes, the majority of the investigated genes were repressed in *H. pylori*. The only notable exception was the predominant up-regulation of *napA* and *vacA* genes in many strains during infection of both AGS cells and PMNs. The following major results were obtained: (i) genetically diverse *H. pylori* exhibit different mRNA expression profiles, (ii) the expression patterns were strain-specific and time-dependent, and (iii) the regulation of expression profiles was host-cell dependent. Our data suggest that contact with target cells leads to an active cross-talk between the pathogen and its host, and that the genetic diversity of *H. pylori* is contributed not only by significant strain-to-strain variation in DNA sequence, but also by the existence of a complex repertoire of coordinated gene regulatory mechanisms in *H. pylori*, which are subject to modulation by the host cells (Gieseler *et al*., 2005).

### *Induction of cellular responses in gastric epithelial cells*

A series of well-studied phenotypical responses elicited during infection of both gastric epithelial cells or phagocytes with *H. pylori in vitro* is shown in Figure 54.2. This includes the induction of motility and dra matic cytoskeletal modifications in gastric epithelial cells, where the *cag*PAI and CagA have been shown to play a role, and the induction of vacuolization and apoptosis by VacA (Montecucco and Rappuoli, 2001; Peek and Blaser, 2002; Backert *et al*., 2002; Amieva *et al*., 2003; Monack *et al*., 2004). The classical activity of VacA in epithelial cells is vacuolization. Several other cellular effects of VacA have also been observed, including: (i) perturbation of vesicle trafficking, (ii) increase in transepithelial ion conductivity, (iii) inhibition of cell proliferation, and (iv) entry into mitochondria followed by cytochrome C release and induction of cell death. It has also been demonstrated that VacA in the presence of CagA plays an important role in the disruption of the epithelial barrier (Amieva *et al*., 2003). Molecular evidence has recently emerged showing that CagA induces a global rearrangement of the host cell actin cytoskeleton, characterized by cellular elongation and cell scattering. First, translocated CagA is phos phorylated on tyrosine residues by Src family kinases on specific EPIYA sequence motifs (Selbach *et al*., 2002). Subsequently, CagA interacts with several cellular

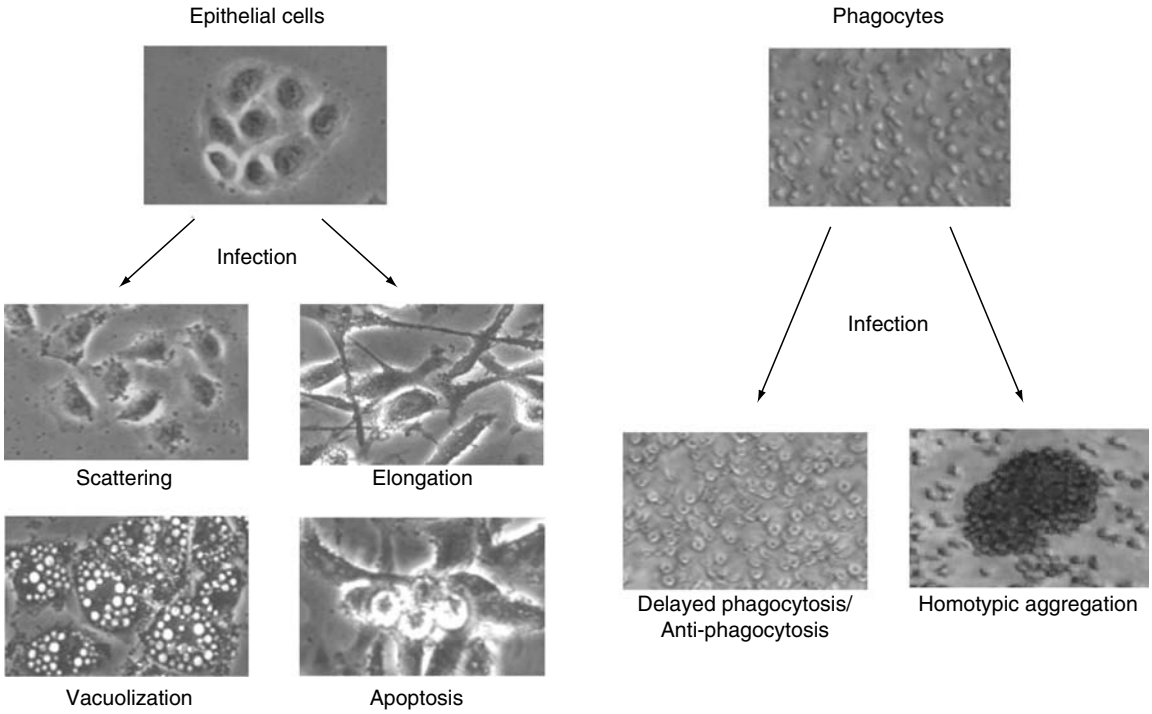



**FIGURE 54.2** Phenotypical responses during *in vitro* infection of AGS gastric epithelial cells and phagocytes with *Helicobacter pylori*.
Infection of AGS cells with *H. pylori* results in different phenotypes that can be observed in a time- and strain-dependent manner. First, AGS infected with *cag*PAI$^+$ *H. pylori* exhibit a strong motility response (after 1–2 hours) followed by elongation of the cells (after 3–4 hours). Vacuole formation can be observed during infection with VacA$^+$ *H. pylori* (after 3–6 hours). Both the *cag*PAI and VacA are also implicated in triggering apoptosis of the infected epithelial cells (after 24–48 hours). Infection of phagocytes by *cag*PAI$^+$ *H. pylori* can induce delayed phagocytosis (Allen *et al*., 2000) or anti-phagocytosis (Ramarao *et al*., 2000) and homotypic aggregation of the infected cells (Moese *et al*., 2002).

proteins in a phosphorylation-dependent or phosphorylation-independent manner (Higashi *et al*., 2002; Mimuro *et al*., 2002) and inhibits the catalytic activity of Src by a negative feedback loop mechanism (Selbach *et al*., 2003). Finally, Src inactivation leads to tyrosine dephosphorylation of the Src substrates ezrin and cortactin (Selbach *et al*., 2003; Selbach *et al*., 2004; Backert *et al*., 2004). Since cortactin and ezrin are key players in regulating the dynamics of the host actin cytoskeleton, this raises the possibility that CagA-induced tyrosine dephosphorylation of these proteins might stimulate actin polymerization via the Arp2/3 complex and N-WASP (neural Wiskott-Aldrich syndrome protein). Indeed, concomitantly with the dephosphorylation events, cortactin relocalizes to the tips of characteristic actin-rich cellular protrusions (Selbach *et al*., 2003). Apart from the reorganization of global actin cytoskeleton, there is also evidence that *H. pylori* can induce local actin polymerization involved in a moderate level of host cell invasion (Kwok *et al*., 2002).

*Helicobacter pylori* is generally considered to be an extracellular pathogen. Until recently, it remained unclear as to how the innate immunity could recognize this pathogen. For example, endotoxin (LPS) is normally a potent inflammatory mediator. LPS of *H. pylori*, however, appears to have a very low biological activity in activating macrophages (Perez-Perez *et al*., 1995). Furthermore, the two *H. pylori* flagellins, FlaA and FlaB, have a markedly low potential to activate TLR5 (Lee *et al*., 2003). The finding that epithelial cells recognize the peptidoglycan-derived GM-tri-DAP muropeptide of *H. pylori* delivered by the type IV secretion system might well be an intriguing answer to the enigma (Viala *et al*., 2004). Based on the fact that a functional *cag*PAI is required for *H. pylori*-induced NF-κB activation in gastric epithelial cells (Covacci *et al*., 1999; Selbach *et al*., 2002), recent data suggest that injected muropeptide by the type IV secretion system is recognized by the intracellular receptor Nod1 (Viala *et al*., 2004). This function of Nod1 in the host defense against *H. pylori* seems to be physiologically important, because Nod1-deficient mice have higher bacterial loads after *H. pylori* infection than do wild-type mice. These results thus extend the function of Nod1 to the detection of both intracellular and extracellular pathogens. Thus, it was proposed that sensing of *H. pylori* by Nod1 could represent a new model for the host recognition of non-invasive or weakly invasive pathogens. In addition, we could recently show that translocated CagA is also able to induce IL-8 release *via* Erk1/2 and NF-κB signaling pathways (Brandt *et al*., 2004). Our results suggest that NF-κB is triggered by multiple pathways, one of which is CagA-dependent. Thus, CagA is a multifunctional protein capable of affecting both actin-remodeling and innate immune responses via the activation of NF-κB.

A major effect of the activation of NF-κB is the induction of the chemokine IL-8 (Viala *et al*., 2004; Brandt *et al*., 2004). The release of IL-8 then attracts inflammatory cells for infiltration into the gastric mucosa. The recruitment and activation of immune cells in the underlying mucosa as part of non-specific immunity are likely to be orchestrated by the combined actions of *H. pylori* muropeptides, epithelial-derived chemokines (e.g., IL-8 and GRO-α), and pro-inflammatory cytokines liberated by PMNs (TNF-α, IL-1 and IL-6) in response to *H. pylori* infection (Bodger and Crabtree, 1998).

### *Modulation of the expression of inflammatory mediators*

The adherence of *H. pylori*, release of VacA, as well as function(s) of the *cag*PAI components such as CagA and peptidoglycan, all contribute to direct epithelial damage and disruption of its barrier function *in vivo* and *in vitro* or to indirect epithelial damage resulting from the host inflammatory response. This damage eventually leads to leakage of nutrients into the mucus layer, which may on the one hand support *H. pylori* growth and survival and on the other hand attract PMNs and other immune cells. For example, it has recently been shown that urease and the *cag*PAI are involved in the induction of transendothelial T cell migration (Enarsson *et al*., 2004). *H. pylori* might be able to come into direct contact with PMNs during infection and epithelial damage. We established an *in vitro* system to study virulence gene expression during interaction of *H. pylori* with these immune cells. In these experiments, we demonstrated that *napA* and *vacA* genes were significantly up-regulated in a number of clinical *H. pylori* isolates during infection of PMNs, a finding that is in agreement with the importance of VacA and NapA proteins for immunomodulatory effects induced by this pathogen (Gieseler *et al*., 2005).

In order to survive in the host, *H. pylori* not only needs to adapt its gene expression pattern, but also has to modulate the host transcriptional profile for its own benefit. Hence, since the regulation of host gene expression by *H. pylori* may play an important role in the development of pathogenesis, we studied the expression of several host cell signaling molecules, including chemokines, NADPH oxidase, lipid mediators, TLRs, and other cell surface receptors in infected PMNs by a variety of methods such as RT-PCR, real-time Taqman-PCR, ELISA, and FACS analyses (Figure 54.3). For example, it was observed that *H. pylori* modulated the expression of CD11b, CXCR2, CD62 ligand (CD62L), and the fMLP receptor in a time-dependent

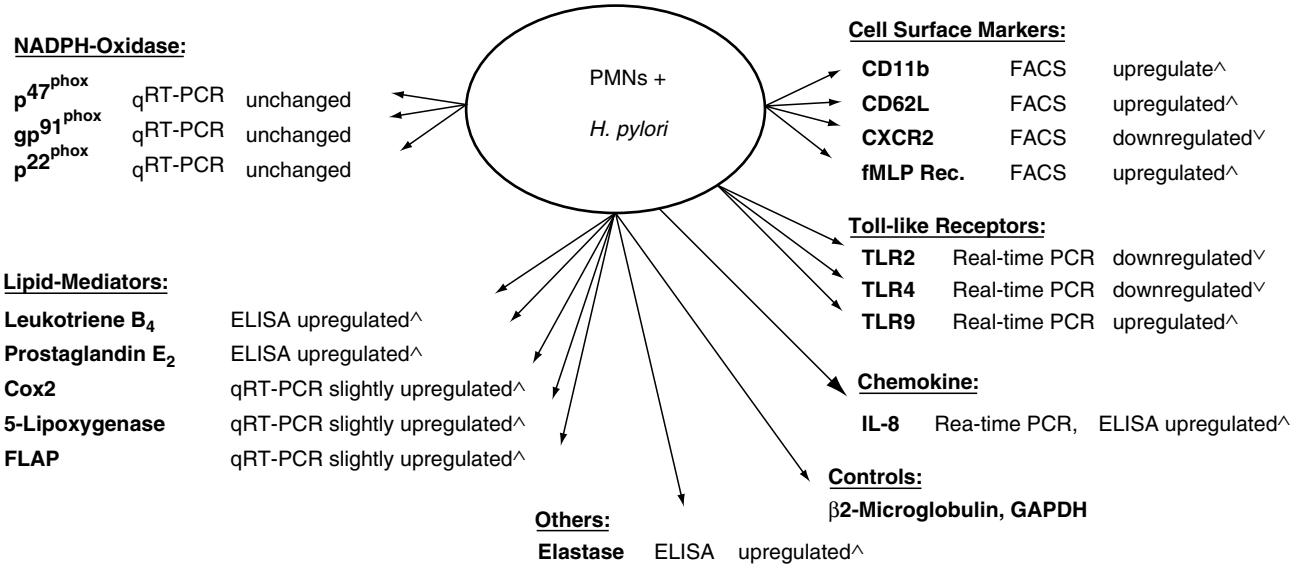



**FIGURE 54.3** Modulation of immune responses by PMNs infected with *H. pylori*.
Several host responses can be quantitated during infection of PMNs with *cag*PAI$^+$ *H. pylori* by FACS, ELISA, RT-PCR, and real-time (Taqman) PCR. The up- and down-regulation of components of the NADPH oxidase complex, lipid mediators, cell surface receptors (including TLRs), and chemokines are indicated.

manner. Stimulation with the specific agonists (fMLP, PMA, or NaF) prior to infection, as compared to stimulation with *Helicobacter* alone, showed marked changes in the immunological parameters, suggesting that the various clinical *Helicobacter* strains exert these events time-dependently but also initiate signal transduction cascades; these either lead to additive, synergistic, or suppressive events. Similar observations were obtained for the *H. pylori*-triggered release of IL-8, TNF-α, $LTB_4$, and $PGE_2$.

The aforementioned changes in chemokine and receptor molecule expression during *H. pylori* infection of immune cells are accompanied by the formation of large homotypic aggregates of infected macrophage-like cells (Figure 54.2). This occurs through up-regulation and recruitment of ICAM-1 to the cell surface of infected cells, which then mediates the aggregation via lymphocyte function-associated molecule 1 (LFA-1), a signaling pathway that may regulate cell-cell interactions and inflammatory responses (Moese *et al*., 2002). However, the actual effector molecule, which appears not to be CagA, remains to be identified.

### *Evasion of the host immune response*

From the evolutionary point of view, the development of severe gastritis and its progression to more serious tissue destruction appears to be against the interest of *H. pylori* to persist in the human stomach. In this context, it is important to note that only a minority of colonized patients indeed develop disease (fewer than 20%), whereas the majority of patients remain largely asymptomatic. Thus, it appears that establishing a balance with the host immune system is one of the major goals for *H. pylori* to ensure a persistent infection. A long-term acquisition of *H. pylori* in the human stomach therefore requires the development of mechanisms to evade killing by the host immune system.

There are a few possible mechanisms for *H. pylori* to impair the bactericidal functions of immune cells. First, *H. pylori* actively retards its uptake by macrophages and subsequently persists inside large vacuoles called megasomes, which result from phagosome fusion (Allen *et al*., 2000). It has been shown that regulation of local actin polymerization and phagocytosis by atypical PKCζ plays a role in the delay of uptake (Allen and Allgood, 2002). Second, in studies using human blood monocytes and PMNs, *H. pylori* has been shown to actively block its own uptake (Ramarao *et al*., 2000). Each of these phenotypes depends on the presence of a functional *cag*PAI secretion system and an yet unknown effector molecule that appears not to be CagA. Third, an arginase (encoded by *rocF*) is expressed by *H. pylori* to down-regulate eukaryotic nitric oxide (NO) production (Gobert *et al*., 2001). The antimicrobial effect of NO is an essential part of innate immunity. The down-regulation of NO thus allows *H. pylori* to evade the immune response.

*H. pylori* has evolved to modulate not only the innate but also the adaptive immune response. The latter is based on MHC-class-II-restricted ($CD8^+$) T cells, and to a lesser degree MHC-class-I-restricted ($CD4^+$) T cells. The *H. pylori* cytotoxin VacA blocks specifically the antigen-dependent proliferation of T cells by interfering with IL-2-mediated signaling (Gebert *et al*., 2003; Bonacristiano *et al*., 2003). This occurs by inhibition of $Ca^{2+}$ mobilization and, subsequently, down-regulation of the activity of the $Ca^{2+}$-dependent phosphatase calcineurin. This in turn inhibits the activation of the transcription factor NFAT (for nuclear factor of activated T cells). In this way, NFAT is kept in a phosphorylated state and hence nuclear translocation is prevented. The target genes, such as IL-2 and the high-affinity IL-2 receptor (IL-2Rα), are, therefore, not expressed. VacA, however, exerts a different effect on human primary $CD4^+$ T cells, whose proliferation is inhibited through the TCR and CD28 (Sundrud *et al*., 2004). In the study of Sundrud and co-workers, VacA is shown to suppress IL-2-induced, cell-cycle progression and proliferation of primary T cells without affecting NFAT. Thus, VacA may inhibit the clonal expansion of T cells that has been activated by bacterial antigens, thereby allowing *H. pylori* to evade the adaptive immune response, resulting in chronic infection. Another possible function of VacA in subverting the adaptive immune response is its ability to interfere with antigen presentation by MHC class II (Montecucco *et al*., 2001).

In antigen-presenting cells (APCs), the processing of bacterial proteins into peptide epitopes is greatly reduced in the presence of VacA, indicating that VacA can abrogate antigen presentation in these cells. The importance of VacA in establishing an infection *in vivo* has been corroborated using animal models (reviewed by Montecucco and Rappuoli, 2001; Peek and Blaser, 2002).

Urease, CagA, VacA, and NapA, apart from being crucial determinants of *H. pylori* colonization and pathogenicity, are also major antigens in the human immune response to *H. pylori* infection. During the past 15 years, these antigens have been examined in numerous animal studies and each of them has been found to elicit immune responses that might either protect from or eradicate a pre-existing infection by *H. pylori*. Clinical trials of test vaccines are underway with the aim of achieving oral or systemic immunization of human patients. If the clinical studies are successful, the chapter on the co-evolution of this pathogen with humans that started more than 150,000 years ago will soon be drawn to an end.

## CYSTIC FIBROSIS

### Inflammatory mechanisms in cystic fibrosis—novel views and approaches

Cystic fibrosis (CF) is the most common fatal genetic disease in white populations (about 1 in 2,500 live births). CF occurs due to recessive mutations in the CF transmembrane conductance regulator (*Cftr*) gene, which encodes the transmembrane chloride channel expressed in the epithelium of multiple organs. Classic CF lung disease presents with a progression of inflammation and infection, and a decline in lung function marked by mucopurulent plugging, bronchiectasis, and intermittent bronchopneumonia (Chmiel *et al*., 2002; Chow *et al*., 2003; Griese *et al*., 2004; Hashimoto *et al*., 2000; Jaeger *et al*., 1992). The role of CFTR in the pathogenic process is widely unclear. It is suggested that the reduction of airway liquid volume results in deficient mucociliary clearance, so entrapped pathogens are not efficiently removed and hypoxic mucous microenvironments encourage the formation of bacterial biofilms (Yu *et al*., 1998; Middleton *et al*., 2003).

In CF, a complex inflammatory reaction of acute and chronic inflammatory mediator generation concomitantly occurs and is decisive for inflammation, immunosuppression, and tissue destruction. Clearly, a multitude of soluble factors, as well as a cross-talk of various cells, determines the clinical outcome of the patients. Early infection of the lungs of patients with CF occurs by *Staphylococcus aureus* or *Haemophilus influenzae*. Later, most patients become chronically infected with *Pseudomonas aeruginosa*, and a minority with *Burkholderia cepacia* complex species (Speert *et al*., 2004). *P. aeruginosa* can convert to a mucoid phenotype, forming biofilms that are highly resistant to conventional antibiotics and are the major cause of respiratory failure in patients with CF. Viruses, non-tuberculous mycobacteria, and fungi may also play a role. A vigorous host response is required to effectively clear the organisms from the lungs. This host defense is dependent on the recruitment and activation of neutrophils and macrophages. It was shown that the CXC chemokines are critical mediators of neutrophil-mediated host defense in *Pseudomonas* pneumonia (Bergmann *et al*., 1989; Dayer *et al*., 1998; Lin *et al*., 2004).

Airway inflammation is a hallmark of CF lung disease. Compared with normal individuals, airway fluids of patients with CF show an increased number of neutrophils and increased levels of the pro-inflammatory cytokines tumor necrosis factor TNF-α, IL-6, IL-8, and $LTB_4$, but decreased levels of anti-inflammatory IL-10. The overproduction of IL-8 is the

probable cause of excessive neutrophil infiltration in the CF lung. Reduced responsiveness to IL-8 of neutrophils from patients with cystic fibrosis is associated with receptor desensitization as a result of exposure to high systemic levels of IL-8 (Cannon *et al*., 2003). Attracted neutrophils, either live or dead, then release noxious compounds, including oxidants, proteinases, and DNA. Additional results showed that peripheral blood neutrophils from normal subjects and individuals with CF contained similar amounts of neutrophil elastase (NE), but after preincubation with CF bronchoalveolar lavage (BAL) fluid, significantly more NE was released by CF peripheral blood neutrophils, a release that was amplified further by incubation with opsonized *Escherichia coli* (Devaney *et al*., 2003; DiMango *et al*., 1998; Taggart *et al*., 2000).

The production of IL-8 and other cytokines in response to *P. aeruginosa* was increased in cells with a CF-like phenotype. However, primary cultures of airway epithelial cells from patients with CF exhibited variable production of IL-8 when exposed to pro-inflammatory stimuli under different culture conditions. It was speculated whether infection is the cause or consequence of the pulmonary inflammation in CF (Moser *et al*., 1997; Muir *et al*., 2004). Several hypotheses have been proposed to explain the predisposition of CF airways to chronic infection. Obviously, CFTR has as a key function an innate immunity to lung infection with *P. aeruginosa*. In addition to its role as an ion channel, wild-type (WT) CFTR acts as a specific receptor for the binding and internalization of *P. aeruginosa* by respiratory epithelia. This ingestion event serves as a protective function against *P. aeruginosa* infection in the normal lung. The steps following internalization of the CFTR/*P. aeruginosa* complex that lead to clearance of the organism are only partially known, and apoptosis could be a key component in both induction of shedding of the infected cells, as well as termination of pro-inflammatory signaling (Pier *et al*., 1997; Wong and Choi, 1997). Regardless of the underlying mechanism, the decreased internalization and delayed apoptosis of cells expressing only mutant CFTR may contribute to the pathogenesis of pseudomonal infection in the patient with CF by allowing for more prolonged bacterial residence time on the lung mucosa, which gives the microbe the opportunity to proliferate and establish infection (Weinrauch and Zychlinsky, 1999; Wolf and Green, 1999). Once established, the chronic infection and inflammatory response does lead to ongoing inflammation and apoptosis in the lung, but it is the failure to initiate and resolve *P. aeruginosa* infection shortly after bacterial exposure within the CF lung that underlies the hypersusceptibility of the patients to *P. aeruginosa* infection.

As the airway epithelium is potentially exposed to a large number of diverse inhaled bacteria, TLRs involved in defense of the airways must be broadly responsive, available at the exposed apical surface of the cells, and highly regulated to prevent activation following trivial encounters with bacteria. These receptor complexes include myeloid differentiation protein (MyD88), IL-1 receptor-activated kinase-1, and TNF receptor-associated factor 6. Recent results indicate that the MyD88-dependent pathway is essential for the development of early host responses to *P. aeruginosa* infection, leading to the clearance of this bacterium, and that TLR2 and TLR4 are involved in this process (Epelman *et al*., 2004; Lorenz *et al*., 2004; Power *et al*., 2004). Consistent with their function in protecting the respiratory tract from inhaled pathogens, there is selective mobilization of TLR2 to the apical surface of the airway cells in response to bacteria, and specifically in response to CF pathogens. However, there is no evidence that TLR-associated signaling is directly affected by CFTR dysfunction.

**Pseudomonas aeruginosa**

It is well accepted that the strategy of *P. aeruginosa* infection allows a change in the expression pattern of pathogenicity factors, which subsequently modulates the pattern of inflammatory mediator induction from human inflammatory effector cells (König B. *et al*., 1996; Schmidt *et al*., 1996). In this regard, we previously showed that *P. aeruginosa* from the early disease stage (acute phase) induced significant amounts of $LTB_4$, release of lysosomal enzymes and histamine from human peripheral cells. *P. aeruginosa* of late-stage disease (chronic phase) was the most pronounced inducer of IL-8, but no longer of $LTB_4$ and enzyme release. During the chronic course of infection, *P. aeruginosa* undergoes an additional conversion from a non-mucoid to a mucoid phenotype. The convergence to the mucoid phenotype of *P. aeruginosa* in CF still seems to be associated with increased inflammation, respiratory decline, and a poor prognosis. This led us to further precisely analyze the bacteria–host cell interaction with the focus on the chemotactic factors IL-8 and $LTB_4$. We selected *P. aeruginosa* strains from CF patients (n = 5) who were colonized by one *P. aeruginosa* strain expressing the non-mucoid and the mucoid phenotype in parallel. The non-mucoid and the mucoid *P. aeruginosa* pairs were subjected to RFLP analysis for clonal characterization. We then analyzed the efficacy of mucoid and non-mucoid *P. aeruginosa* to induce neutrophil migration in a transwell system. In this regard, mucoid *P. aeruginosa* strains dose-dependently attracted up to 20% of the neutrophils during a defined time interval, as

compared to non-mucoid strains with a maximum of 6% attracted neutrophils (Figure 54.4A).

In parallel, the expression of CXCR2, one of the two IL-8 receptors on neutrophils, was markedly down-regulated on neutrophils migrating towards *P. aeruginosa* bacteria expressing the mucoid phenotype, indicating the pronounced response of the neutrophil and also its activation. The results for CXCR1/CXCR2 expression were confirmed by FACS analysis as well as by quantitative RT-PCR (Figure 54.4B). The more pronounced down-regulation of CXCR2 by the mucoid *P. aeruginosa* bacteria, as compared to the non-mucoid *P. aeruginosa*, suggests autocrine or paracrine differences in cell surface regulation of chemokine receptors and IL-8 release. Clearly, the various chemotaxins differ in their effect on CXCR1 and CXCR2 surface expression on PMNs. In comparison to PMN migrating towards $LTB_4$, neutrophils attracted by IL-8 revealed a more pronounced down-regulation of CXCR2 surface expression, indicating the specificity for the receptor and the appropriate chemokines.

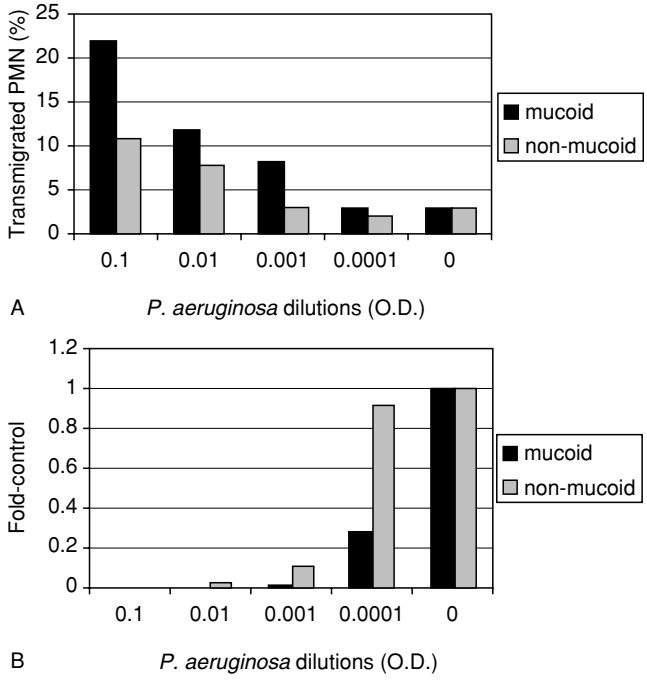



**FIGURE 54.4** Neutrophil CXCR2 mRNA expression in *P. aeruginosa* attracted neutrophils.
In a Transwell system with a 5fm-pore-size polycarbonate filter, the upper compartment was filled with neutrophils ($2\times10^5$ in 200µl), the lower compartment contained 800µl of control buffer or the respective dilutions (optical densities at 600nm of 0.1, 0.01, 0.001, 0.0001) of *P. aeruginosa* bacteria expressing either the mucoid or the non-mucoid phenotype. The incubation proceeded for 4h at 37°C in a $CO_2$ incubator. **A)** The migrated cells in the lower compartment were counted and **B)** were submitted for quantitative real-time RT-PCR. CXCR2 mRNA expression was quantified as CXCR2-specific copies per neutrophil, and data were expressed as stimulation index as compared to untreated neutrophils. Data was the mean of 4 independent experiments.

The epithelial-endothelial link is a key regulator of neutrophil sticking, extravasation, and migration. In this regard, it has been shown that epithelial cells by their complex strategy of chemokine induction activate endothelial cells for neutrophil migration and activation. Our studies extended this current view by using a transendothelial transwell system. For this purpose, transwell chambers were coated with human umbilical endothelial cells, and the transendothelial migration of indicator neutrophils was studied with regard to mucoid versus non-mucoid *P. aeruginosa* strains. A concentration-dependent transmigration of neutrophils was obtained, which was again more pronounced for mucoid than non-mucoid *P. aeruginosa* strains. This correlated again with a pronounced down-regulation of the CXCR2 mRNA expression in response to the mucoid, as compared to the non-mucoid *P. aeruginosa* strain. As has been shown in the past by previous investigators, the bactericidal activity of neutrophils is governed by the functional activity and complexity of the NADPH oxidase complex. Consequently, the activation of granulocytes induces the up-regulation of the various components of the NADPH oxidase complex, leading to a functional respiratory burst. Under normal conditions, the bactericidal activity is of major importance for the killing of bacteria; it also has adverse effects once it is directed toward the adjacent tissue. This is obviously valid in the deleterious process of CF disease.

In a set of further experiments, we analyzed the $p22^{phox}$ and $p47^{phox}$ mRNA expression in neutrophils after their transendothelial migration towards mucoid and non-mucoid *P. aeruginosa* bacteria. For comparison, we used PMN after their transendothelial migration toward the chemotactic factors IL-8, $LTB_4$, and C5a. It could be clearly demonstrated that the chemotactic factors differ in their activation pattern of the membrane-bound $p22^{phox}$ subunit and the cytoplasmic $p47^{phox}$ subunit of the NADPH oxidase complex. IL-8 induced an up-regulation of $p22^{phox}$ and $p47^{phox}$ mRNA expression only at high concentrations (10–100ng), $LTB_4$ over a wide concentration range (0.1–100 ng/ml), and C5a revealed nearly no $p22^{phox}$ or $p47^{phox}$ mRNA expression. A comparison of *P. aeruginosa* mucoid versus non-mucoid strains clearly indicated that the mucoid strain over a wide concentration range induced a significant expression of $p22^{phox}$ and $p47^{phox}$ mRNA, unlike the non-mucoid strains (Figure 54.5). This again indicates differences in the functional activity of PMN exposed towards mucoid versus non-mucoid strains and suggests a potent activity of the mucoid strains on the NADPH oxidase complex.

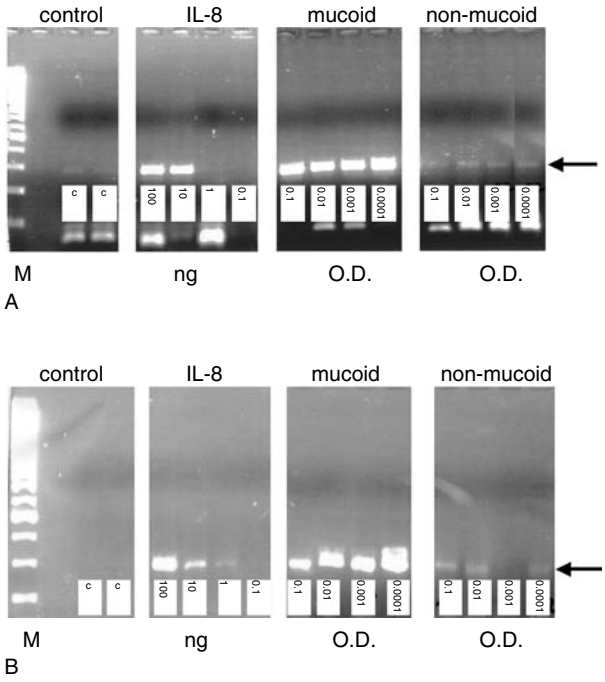



**FIGURE 54.5** Activation of the NADPH complex by *P. aeruginosa*. In a Transwell system, the 5µm-pore-sized polycarbonate filter was overlayered with endothelial cells (human umbilical endothelial cells) and the upper compartment was filled with neutrophils ($2\times10^5$ in 200µl), the lower compartment contained 800µl of control buffer (c) or the chemokine IL-8 (100-, 10-, 1-, 0.1ng) or bacteria from a *P. aeruginosa* isolate, expressing either the mucoid or the non-mucoid phenotype. The bacteria were used at different concentrations, which were determined via the optical density (O.D) at 600nm. The incubation proceeded for 4h at 37°C in a $CO_2$ incubator. The migrated cells in the lower compartment were counted and were submitted for qualitative RT-PCR. A) $p22^{phox}$ mRNA expression; B) $p47^{phox}$ mRNA expression. The results from one typical experiment are shown.

TLRs are major sensors of microbial recognition, as well as soluble exoproducts. With regard to the CF disease process, a major role has to be attributed to the expression of TLR2, 4, and 9. Quite recently, it was shown by the use of microarray analysis that upon *P. aeruginosa* conversion to mucoidity, there is a massive and preferential induction of genes encoding bacterial lipoprotein. Bacterial lipoproteins are potent agonists of TLR2 signaling. In parallel, the authors could show an increased expression of TLR2 in human respiratory epithelial cells as was ascertained by Western blot analysis. Thus, the excessive inflammation in CF is the result of a global induction in mucoid *P. aeruginosa* of lipoproteins that act as pro-inflammatory toxins superimposed on the hyperexcitability of CF cells. Moreover, bacteria can alter the acylation state of their LPS in response to environmental changes. *P. aeruginosa* synthesizes more highly acylated (hexa-acylated) LPS structures during adaptation to the CF airway. Therefore, a change in the recognition patterns by TLRs occurs. Although one failed to detect a global effect of CFTR dysfunction on TLR expression in the lung, the participation of TLR2 in airway cell responses to bacteria has been appreciated. Both *S. aureus* and *P. aeruginosa* stimulation appeared to be signaled through TLR2, despite the presence of TLR4 in these cells. The availability of TLR2 on the apical surface of respiratory epithelial cells suggests a role for this TLR in airway responses to Gram-negative as well as Gram-positive pathogens (Soong *et al*., 2004). The participation of specific TLRs in airway cells clearly differs from what is observed in cells of hematopoietic origin. TLR4, which is critical in signaling systemic responses to LPS, was substantially less involved in epithelial signaling, although clearly present in the epithelial cells (Yamamoto *et al*., 2002). Airway epithelial cells, like other mucosal epithelia, are not particularly responsive to LPS, as compared with myeloid cells. We investigated the quantitative TLR mRNA expression in human neutrophils using real-time Taqman RT-PCR. Neutrophils expressed high levels of TLR2-specific mRNA, ranging donor-dependently from $10^6$ up to $1.5\times10^7$ copies/$10^6$ neutrophils (n = 12). TLR4-specific mRNA was detected to a lesser degree with a mean of $5.4\times10^3 \pm 3\times10^3$ copies/$10^6$ neutrophils (n = 14). TLR7 and TLR9 mRNA expression were barely detected with less than $10^2$ copies/$10^6$ neutrophils. Additionally, we confirmed the expression of TLR2 and TLR4 at the protein level. In this regard, neutrophils showed significant higher signals for surface TLR2 as compared to surface TLR4. Therefore, TLR2 seems to play a predominant role in the innate immune response of neutrophils.

In addition, we analyzed to what extent the migration of human neutrophils towards $LTB_4$, C5a, or IL-8 is accompanied by changes in TLR2 mRNA expression. Obviously, these chemotaxins are abundantly generated and released in CF. Our results showed that the chemotactic factors differ markedly in their effects on TLR2 mRNA expression. Transmigrated neutrophils towards IL-8 (from 0.1 up to 100ng) showed an upregulation of TLR2, which was most prominent at the highest concentrations. An opposite dose dependency is obtained with $LTB_4$, reflecting low TLR2 expression at the highest concentration of $LTB_4$ (100 ng), as compared to the lowest concentration (0.1 ng/ml) (Figure 54.6). Whether these differences in TLR2 expression towards the two key chemokines IL-8 and $LTB_4$ reflect the increased inflammatory response seen in the chronic CF airways has to await further studies.

We extended our studies using additional chemokines such as GCP-2 GRO-α and NAP2, as well

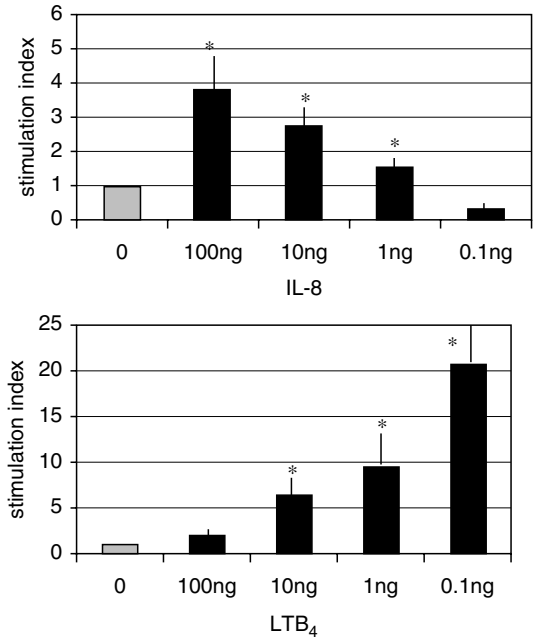



**FIGURE 54.6** Neutrophil TLR2 mRNA expression in chemoattracted neutrophils.
In a Transwell system with a 5µm-pore-size polycarbonate filter, the upper compartment was filled with neutrophils ($2\times10^5$ in 200µl), the lower compartment contained 800µl of control buffer or the chemoattractants IL-8 or $LTB_4$ at the indicated concentrations (100-, 10-, 1-, 0.1ng). The incubation proceeded for 4h at 37°C in a $CO_2$ incubator. The migrated cells in the lower compartment were counted and were submitted for quantitative real-time RT-PCR. TLR2 mRNA expression was quantified as TLR2-specific copies per neutrophil, and data were expressed as stimulation index as compared to untreated neutrophils. Data were the mean ± SEM of 10 independent experiments, with significant differences in TLR2 mRNA expression, as compared to spontaneously migrated neutrophils indicated: **, $p < 0.01$; *,$p<0.05$

as ENA-78, which are released from different cell sources during inflammation after immunological and non-immunological activation of different cells. The TLR2 mRNA copies per transmigrated neutrophils were studied. Obviously, over a wide concentration range (0.1 up to 100ng), the chemotactic factors respond differently with regard to the induction of Toll-like receptor 2 expression showing the highest activity for ENA-78>NAP2>GRO-α>GCP-2>IL-8. In comparison, IL-8 has the highest chemotactic potential, followed by GCP-2 and GRO-α with similar chemotactic activities and by NAP-2. ENA-78 expressed the weakest chemotactic activity towards neutrophils in a transwell system. Thus, a reverse pattern of TLR2 expression versus chemotaxis is obtained for the transmigrated neutrophil. However, in a complex scenario of microbial-cell interaction the different maturation stages of granulocytes, as well as their individual variations in functional activities and quite recently the polymorphism of TLRs, have to be considered and are described (Beutler, 2004; Kaufmann *et al*., 2004; Maggi, 1998).

Taken together, these data clearly indicate that the neutrophil effector function is not only modulated by microbial pathogenicity factors and/or toxins, but also by individual chemokines. Obviously, the scenario of CF is even more complex since the disease process is further complicated by, e.g., microbial biofilm formation. In addition, the impact of antibacterials on parameters of the non-specific immune system has become of increasing interest (Van Vlem *et al*., 1996). Novel data have been raised for the effect of fluoroquinolones (Ciprofloxacin, Moxifloxacin) on various cell functions including neutrophils. Therefore, we analyzed the influence of the fluoroquinolone moxifloxacin on the migration of neutrophils towards the chemotactic factors IL-8, $LTB_4$, and C5a. The choosen concentrations of moxifloxacin (from 0.08 up to 80mg) were within the physiological concentrations achievable in patients (König, B. *et al*., 2004; Soman *et al*., 1999). The presence of moxifloxacin resulted in an increased migration of PMNs towards IL-8 by up to 166±8%, but suppressed the migration towards $LTB_4$ down to 1.15±0.87% and showed no effects with regard to C5a. In parallel, moxifloxacin down-regulated the TLR2 mRNA expression in PMN, migrating towards IL-8 by approximately 90% and up-regulated TLR2 mRNA expression in PMN migrating towards $LTB_4$ up to 300%. Further data indicate that antimicrobials also affect toxin release to a various degree from viable bacteria, which modulate host defense strategies (Aoki *et al*., 1994; Fischer and Adam, 2001; Gemmell, 1993; Labro, 1993; Mueller *et al*., 1999). In the context of CF, these studies are necessary to benefit from a precise time-scheduled use of antimicrobials such as the fluoroquinolones.

### Staphylococcus aureus

*S. aureus* is often the first pathogen to infect the respiratory tract of CF patients. In the pre-antibiotic era, *S. aureus* and *H. influenzae* caused substantial morbidity and mortality in infants with CF, but with the advent of antibiotic therapy effective against these pathogens, the life expectancy of infants with CF has increased. However, the eradication of *S. aureus* apparently favors the colonization with *P. aeruginosa*, suggesting a protective role of *S. aureus* in the early disease process. The later disease stages are complicated by the presence of small colony variants of *S. aureus* (Kahl *et al*., 2003).

#### *The role of superantigens in pathogenesis*

*S. aureus and Streptococcus pyogenes*, as well as a few other bacteria and viruses, produce and secrete a group

of exotoxins called superantigens (SAGs) (Becker *et al*., 2003; Hensler *et al*., 1993; Saloga *et al*., 1996; Silverman, 1997). SAGs have received a great deal of attention since the discovery of their mechanisms in 1989. At least 20 SAGs (or molecules with SAG-like activity) have been identified, and are synthesized by the bacteria that occur in the gut, at least sporadically. The most well-characterized SAGs are the staphylococcal enterotoxins (SE) A, B, C (antigenic variants C1–3), D, E, and the recently discovered enterotoxins G to Q, and toxic shock syndrome toxin-1 (TSST-1), as well as the streptococcal SAGs, including the pyrogenic exotoxins (SPEs and antigenic variants A1–4), C, G–J, L and M, streptococcal mitogenic exotoxins (SMEZ), and the streptococcal SAG (Baker and Acharya, 2004; McCormick *et al*., 2001; Petersson *et al*., 2004). It appears that each strain of these pathogens produces a distinct set of SAGs, but not all different types of SAGs. Structural studies over the last 10 years have provided a large amount of knowledge regarding the complex interactions of these molecules with their receptors. This knowledge, combined with the wealth of new information from genomic and biochemical studies as well as the availability of crystal structures, has shown the molecular architecture of some toxins and revealed a variety of molecular mechanisms of action (Li *et al*., 1998; Li *et al*., 2001; Papageorgiou *et al*., 1996).

SAGs bind specifically to both APCs and MHC class II molecules. Unlike conventional antigens, SAGs remain unprocessed by the APCs, instead binding direcly on the outside of MHC class molecules. Here, cross-linking occurs to both MHC class II α and β chains (Campbell and Kemp, 1997; Dinges *et al*., 2000; Drynda *et al*., 1995; Hofer *et al*., 1995; Mueller-Alouf *et al*., 1996; Mueller-Alouf *et al*., 1997; Petersson *et al*., 2001; Petersson *et al*., 2003). The existence of two distinct MHC class II binding sites enabled the formation of a trimeric SEA-MHC-SEA complex as observed in solution experiments. Almost all known SAGs interact exclusively with the Vβ region of the TCR, resulting in T cell stimulation. In this context, it is important to note that while investigating the TCRV profile for SEH toxin, it was discovered that in contrast to all other SAGs, it activated T cells via $TCRV_{\alpha}$ and independent from TCRVβ (Petersson *et al*., 2003).

Recently, we described the effect of leukocidin and various superantigens on innate as well as adaptive immunity. We showed that the composition of the binary toxin leukocidin determines the potency of inflammatory reactions, as well as the down modulatory activity of the granulocyte in response to stimulatory chemotactic factors (König B. *et al*., 1997; König B., *et al*., 1995; Prévost *et al*., 1995). With regard to the various SAGs, we showed that mutant proteins as well as peptides were able to skew the T effector function, as well as initiate and modulate the cytokine production from various human effector cells (e.g., TNF-α, IL-8, IL-10, IL-12) (Drynda *et al*., 1995; Neuber *et al*., 1991; Veldkamp *et al*., 2000).

Although the presence of *S. aureus* has also been documented as a common pulmonary pathogen in CF, there is similarly little or no information on the possible role of staphylococcal SAGs in this disease. Moreover, quite recently, methicillin-resistent *S. aureus* (MRSA) were observed in CF patients. Obviously, MRSA reflects a changing epidemiology (Lina *et al*., 2003; Vandenesch *et al*., 2003). In this regard, a dynamic occurrence of methicillin-resistant *S. aureus* strains has been observed, including the appearance of community-acquired MRSA expressing distinct pathogenicity profiles (Novick *et al*., 2001; Tiemersma *et al*., 2004). About 200 consecutive MRSA recovered during a one-year period were analyzed (Ghebremedhin *et al*., 2004). We determined the antibiotic susceptibility: The isolates were typed by multilocus sequencing (MLST), by a multiplex PCR for the staphylococcal cassette chromosome *mec* (SCC*mec*), by allelle-specific PCR for the staphylococcal accessory gene regulator (*agr*), and by PCR for the presence of toxin genes (*sea–sej*, *tsst-1*, *hlgA, C, B*, *lukE/D*, *luk-PVL*) (Enright *et al*., 2000; Enright *et al*., 2002; Gouaux *et al*., 1997; Jarraud *et al*., 2002; Jarraud *et al*., 2001; Oliveira and Lencastre, 2002; Omoe *et al*., 2002). All strains possessed the genes for *seg* and *sei*. Besides *seg* and *sei*, 119 *S. aureus* isolates were found to be positive for one or more SE genes. Amoung them, 33 strains were positive for *sea*, one for *seb*, 58 positive for *sec*, 27 for *sed*, none for *see*, *seh*, and *sej* gene. No MRSA strain expressed genes for the superantigens ETA, ETB, or TSST-1. Only 12 strains harbored two additional SE genes. With regard to the leukocidins, all strains were positive for *hlgA-hlgB/hlgC-B* and for *lukD/E*. Only eight isolates were positive for the Panton-Valentine leukocidin gene. These results clearly reflected the heterogeneity of *S. aureus* and in particular MRSA with regard to their pathogenicity profile expression. Thus, we have to consider a similar heterogeneity of MRSA as well as of MSSA in CF patients. With regard to the *agr* type, recent data have been described by Kahl *et al*., (2003).

Among the multitude of actions on immune effector cells by superantigens (cytokine induction, T cell skewing, IgE antibody synthesis), recent data have focused on their role on chemokine receptor expression. Adaptive immunity has been considered to be either Th1 or Th2 regulated in cooperation with T regulatory cells. It has been described that Th1 and Th2 cells express a defined set of chemokine receptors. Staphylococcal SAG producing *S. aureus* can be frequently

isolated from the skin of patients with atopic dermatitis. Epicutaneous application of intradermal injection of staphylococcal enterotoxins induces inflammation of the skin, which is T cell dependent. The interaction between chemokines and chemokine receptors (CKRs) is an important step in the control of T cell migration into sites of inflammation (Chambers, 2001; Leung, 1998; Lina *et al*., 1999; Neuber *et al*., 1992; Saloga *et al*., 1996).

Our studies were focused on the role of the staphylococcal superantigen B on chemokine receptor expression. Human peripheral blood mononuclear cells were used as target cells, as well as Jurkat T cells. We systemically investigated the association of 14 CKRs (CCR1-9, CXCR1-5) with CD4+ T cells from peripheral blood mononuclear cells (PBMC) with or without SAG staphylococcal enterotoxin B (SEB) stimulation. CKRs expression on CD4+ T cells from PBMC were analyzed by flow cytometry and RT-PCR. Cytokine secretion was assessed by ELISA in the supernatants from SEB-stimulated PBMC. SEB increased the surface antigen expression (CD45RO, CD31, CD25, CD69, CD54, and FAS) and induced the cutaneous lymphocyte-associated antigen (CLA) on T cells, the homing receptor for skin T cells. A wide range of CC-chemokines were produced by SEB-treated PBMC. These included MIP-1α, MIP-1β, MCP-1, eotaxin, and RANTES, which bind the chemokine receptor CCR3-5. In addition, peripheral cells of these patients showed a pronounced hyper-releaseability of chemokine release and inflammatory mediators (Neuber *et al*., 1991). SEB preferentially up-regulated CCR3, CCR4, CCR5, and CXCR3 on the surface of CD4+ T cells from normal individuals. The maximum effects were observed after 5–7 days of incubation. Furthermore, PBMC were polarized towards Th1 or Th2 cytokine-expressing T cells after incubation in the presence of IL-12/anti-IL-4 or IL-4/anti-IL-12. In polarized Th2 CD4+ T cells, SEB up-regulated CCR4 (Th2-associated CKRs), but suppressed CCR3. Similarly, SEB induced an up-regulation of CCR4-mRNA on isolated CD4+ T cells from atopic donors. CXCR3 (Th1-associated CKRs) was found to be expressed at high levels on Th0 cells after SEB stimulation. In polarized CD4+ T cells, SEB led to a slight down-regulation of CXCR3 (Th1-associated CKRs).

Our results thus demonstrate that SEB modulates CD4+ T cells with regard to chemokine release and chemokine receptor expression. Furthermore, SEB influences Th1- and Th2-associated chemokine receptors. A dynamic pattern of chemokine receptor gene expression may control the tissue-specific migration of effector cells and thus trigger Th1 and/or Th2 immune effector reactions.

### *Emerging microorganisms*

Long-term survival of CF patients is improving due to a better understanding of the basic underlying defects. Some of the common pathogens in CF have led to new treatment strategies, including antibiotic prophylaxis, prevention of colonization of the lungs with *P. aeruginosa*, and the development of new drugs and methods of drug delivery, such as nebulized antibiotic therapy. Difficult new issues, in addition to the increasing pattern of antibiotic-resistant organisms (e.g., MRSA), include the emergence of a number of microorganisms whose relevance in CF is not yet clear. In focus are the non-tuberculous mycobacteria and also respiratory viruses.

#### *Non-tuberculous mycobacteria (NTM)*

In the past years, an increasing prevalence of NTM colonization among the CF population was observed (König, B. *et al*., 2004). Various studies suggest that NTM play an important role in the impairment of pulmonary disease in patients with CF. In this regard, at the case report level, *Mycobacterium abscessus* has been associated with poor outcomes. Mainly due to the misidentification of *M. abscessus*, its role for the clinical outcome in CF is not yet clear. We studied 1,700 sputum samples of CF patients for the presence of NTM. We detected 57 *M. abscessus* isolates belonging to seven CF patients. A correct identification was obtained by sequencing the *hsp65* gene or the 16S-23S intergenic sequence. Our data showed distinct *M. abscessus* strains among the CF patients. Thus, the future challenge is to consider the clinical relevance and proper treatment of NTM species, especially of *M. abscessus* in CF patients and also their contribution to toxin-mediated lung infection.

#### *Respiratory viruses*

Several studies suggest that some patients with CF may be at increased risk of respiratory virus infections that have pronounced and long-lasting effects on those patients, resulting in significant decline in lung function, increasing both the frequency and duration of hospitalization (Abman *et al*., 1988; Armstrong *et al*, 1998; Black *et al*., 1998; Hiatt *et al*., 1999; Zhang *et al*., 2002). Deleterious effects on patients with CF have been reported for most viruses studied, but the effects of respiratory-syncytial-virus (RSV) and influenza are most severe. Epidemiological, viral, and host factors account for the severity. With regard to the latter, production of nitric oxide, an important part of the inflammatory response to viral infections, is reduced in infants with CF. We analyzed about 300 RSV isolates from four associated study centers (Hamburg, Bochum, Dresden,

Freiburg) during the study year 1999–2000 with regard to their genetic relatedness by sequencing the first hypervariable region of the RSV-G-protein. The circulating RSV strains could be divided into three clusters. There was a significant correlation between RSV cluster 2 and disease severity (Struck *et al*., 2004). The mechanism of pathogenicity has to be elucidated.

Quite recently, the human Metapneumovirus was first isolated from children in the Netherlands. The HMPV belongs to a newly discovered virus from young children with respiratory tract disease (see König, B. *et al*., 2004). HMPV produces RSV-like illness in children, ranging from upper respiratory tract disease to severe bronchiolitis or pneumonia. We tested in the population-based prospective PRI.DE multicenter study (Germany, two years) 3,369 nasopharyngeal secretions from children under three years of age with lower respiratory tract infections (LRTI) for RSV A and B, influenza viruses (IV) A and B, and parainfluenza viruses (PIV) 1–3 (König B. *et al*., 2004). Of children requiring intensive care ($n$ = 85), 18% had HMPV, 60% of them in combination with RSV. In a randomly selected subset of RSV-positive nasopharyngeal secretions (n = 120) from children not requiring intensive care support, we did not detect hMPV. In virus-negative samples from patients without intensive care support (n = 620) hMPV was detected in fewer than 1%. Our data support the hypothesis that RSV and hMPV together are more severe than either RSV or hMPV alone, at least in non-CF children below the age of three years. A role of HMPV alone or in combination with RSV in the CF population has to be considered. Furthermore, there is substantial evidence that respiratory virus infections may facilitate bacterial infections. In this regard, the appearance of *Staphylocoocus aureus*, producing the Panton-Valentine leucocidin (Luk-PVL) in influenza-infected children leads to a fatal outcome (Krell *et al*., 2003).

We focused on RSV since this virus is the most common cause of viral infection in infants and children. To develop a better understanding of how RSV infects the lung and of the pathophysiological consequences of viral infection, we investigated the respiratory virus-epithel cell interactions. The F and G proteins are two largely glycosylated proteins expressed on the surface of the viral envelope (Arnold *et al*., 2004; Arnold and König, 2005; Arnold and König, 2005).

At present, there is a concern to use the RSV in gene replacement therapy in CF. For the development of strategies to deliver therapeutic transgenes to the airways of patients with diseases such as CF, it is of fundamental importance to exploit the mechanisms that RSV has evolved to infect the respiratory epithelium. This then might allow us to reduce the recurrent bacterial infection and the toxin-induced destruction of the lung tissue.

### *Microecology*

As long as gene therapy is not yet available, antimicrobial treatment must be optimized, along with measures for the prevention of early infection and the transmission of the pathogens among CF patients. These measures largely rely on the knowledge of the microbial status of the CF patient at all times. CF is dominated by the presence of microbial biofilm formation; the expression and release of microbial pathogenicity factors and toxins are modulated by the cooperative interaction of the microorganisms. Molecular biological methods seem to be appropriate for a more precise analysis of microbial pathogenicity.

Recently, we described the so called T-RFLP method (**T**erminal **R**estriction **F**ragment **L**ength **P**olymorphisms) for the complete qualitative and quantitative analysis of the respiratory microecology (Trotha *et al*., 2002). The method allows us to track static and dynamic changes in the microbial communities under antibiotic therapy; thus, a better knowledge of interactions between commensal and/or pathogenic microbes with each other and with the human host is obtained. We used model microbial communities consisting of CF relevant microorganisms, e.g., *P. aeruginosa, S. aureus, Klebsiella pneumoniae, Burkholderia cepacia, Stenotrophomonas maltophilia, Alcaligenes xylosoxydans*, and *Mycobacterium abscessus*. Fifty-one sputum samples from 15 patients suffering from cystic fibrosis at different stages of disease and antibiotic therapy were analyzed. Our extended studies showed the changes in the microbial pattern after antibiotic treatment (Figure 54.7). This technique will also allow us to determine the fluctuating pattern of microbial communities, the formation of biofilms, as well as of the regulation of bacterial toxin production. In this regard, the virulence of *S. aureus* is dependent on the temporal expression of a diverse array of virulence factors, including both cell-associated products, such as protein A, collagen- and fibronectin-binding protein, and secreted products including lipases, proteases, alpha-, beta-hemolysin, and the respective superantigens. We studied to what extent *P. aeruginosa* is able to modulate the expression of staphylococcal SAGs, e.g., enterotoxin A. Both microorganisms were co-cultured over various times, and the expression pattern of staphylococcal SEA-specific mRNA was analyzed, representative for the classical superantigens. We have clearly shown that the presence of *P. aeruginosa* at increasing concentrations down-regulated the SEA-mRNA expression of *S. aureus* prior to a decrease of the colony-forming units of *S. aureus* (Figure 54.8).

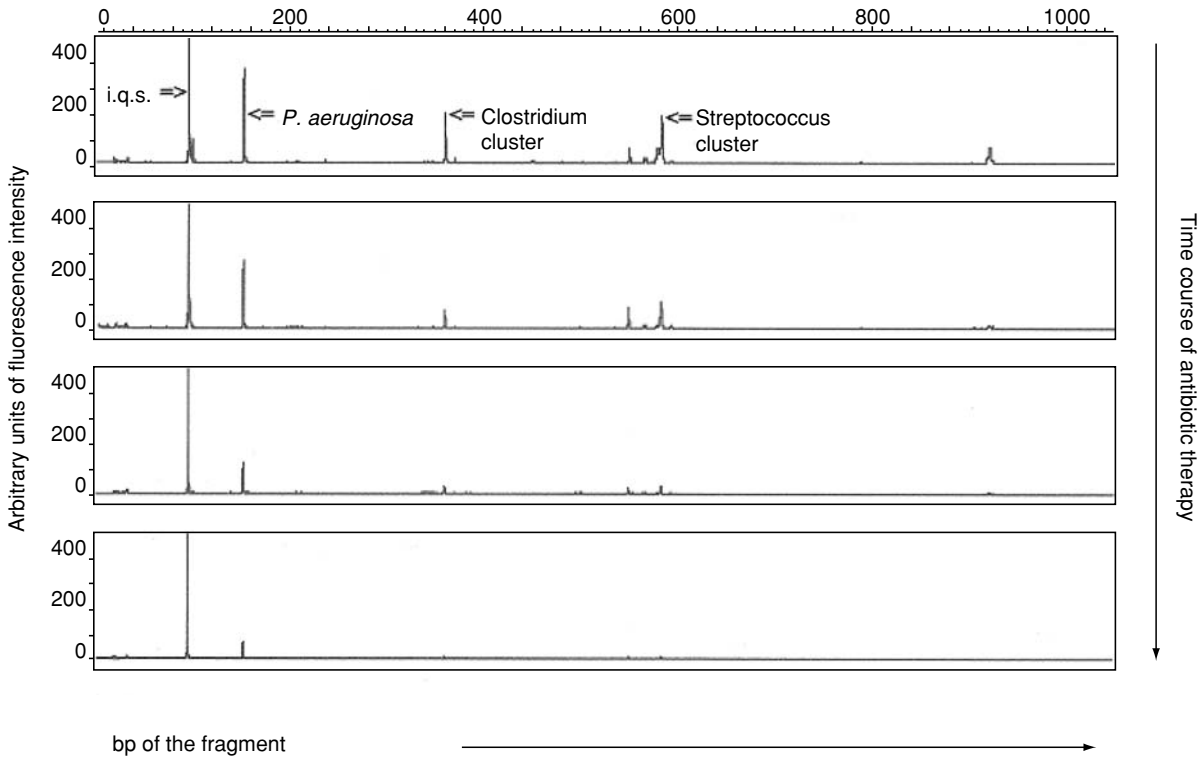



**FIGURE 54.7** Therapy-related changes in the T-RFLP pattern of CF sputum samples.
The T-RFLP pattern in the sputum samples from one individual CF patient at the beginning of antibiotic therapy and at 3, 9, and 14 days after the onset of antibiotic therapy was determined. The 155 bp peak for *P. aeruginosa*, as well as the peaks for the *Clostridium and Streptococcus ssp*. clusters, are decreasing during antibiotic therapy. The 98 bp internal quantification standard is not influenced by the antibiotic therapy

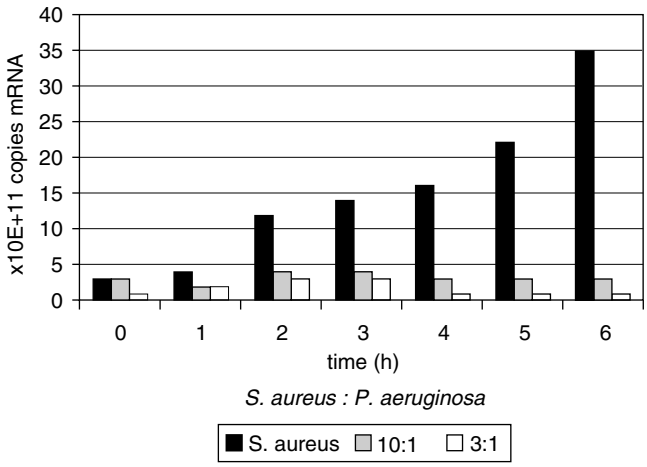



**FIGURE 54.8** *Pseudomonas aeruginosa—Staphylococcus aureus* interaction.
*S. aureus* bacteria were cultivated in the absence or in the presence of *P. aeruginosa* PAO1 (10:1; 3:1) in a total volume of 10ml for up to 6h in Lydia broth medium at 37°C with gentle agitation. After the indicated time intervals, a) the colony forming units of *S. aureus* as well as of *P. aeruginosa* were determined; b) total RNA was prepared and the staphylococcal enterotoxin A specific (SEA) mRNA copies were determined by quantitative real-time RT-PCR. Values represent SEA-specific mRNA copies per ml culture.

At present, we focus on multimicrobial cultures by applying chemostat procedures for biomodeling purposes. Mathematical models will predict the microbial disease status, the pathogenicity profile of the relevant microorganisms, and the efficacy of treatment in CF patients, but also in other infectious diseases. Thus, toxin-mediated cellular and cell-biological events can be exactly quantitated and evaluated in the future.

## ROLE OF BIOPROSTHETIC DEVICES AND BACTERIAL EXOPRODUCTS / TOXINS

Biomaterials implanted for many months or years must meet two criteria: First, they must have specific mechanical properties to replace the functions of defective body tissues or organs, and, second, they must be accepted and integrated by the host in a controlled and predictable way. No implanted artificial material can be considered totally inert in the body and can lead to aseptic and furthermore septic inflammation. The incidence of infections ranges from 1–25% and are mostly attributed to *S. aureus* and/or *S. epidermidis*.

## Biopolymers and inflammation

The mechanisms of a variable host response towards different categories of biomaterial implants are complex and still poorly understood. Depending on the circumstances, some of these materials can elicit a chronic inflammatory response, also called a foreign-body reaction, which has the following characteristics: After an initial acute inflammatory reaction, a chronic granulomatous tissue reaction may persist, even after encapsulation has occurred. The foreign-body reaction seems to be induced by continuous chemical or mechanical stimuli arising from the biomaterial implants. Morphological analysis of this reaction reveals the presence of a large number of macrophages, which generally attempt to phagocytize the material.

Some of the macrophages then merge their cytoplasm to become multinucleated giant cells, also called foreign-body giant cells. If the foreign body cannot be degraded by phagocytes, granulation tissue is formed to encapsulate the foreign body and to isolate the implant from the rest of the body tissues. The foreign-body reaction is further characterized by the presence of the inflammatory cells, namely, the polymorphonuclear leukocytes (Cross and Welsh, 2001; Waldvogel and Bison, 2000). Activation of phagocytes that come into contact with the non-phagocytosable foreign material leads to the secretion of inflammatory mediators, including acidic or neutral hydrolases, activated complement components, TNF, interleukins, prostaglandins, plasminogen activator, and coagulation factors. These secreted factors interact in a complex manner to control and maintain acute and chronic phases of the inflammatory response to implants. Materials in contact with blood must fulfill additional requirements; they must not damage blood cells and/or encourage the formation of blood clots (Waldvogel and Bison, 2000).

Infections associated with indwelling medical devices are caused predominantly by staphylococci. These infections are initiated by bacteria adhering either directly to the device prior to implantation or to host proteins that coat the implant surface after implantation. The first mechanism is considered to be most important for *Staphylococcus epidermidis*, while *Staphylococcus aureus* infection is thought to be mediated by protein adhesions on the bacterial cell surface that recognize fibrinogen and fibronectin among the host proteins that rapidly coat biomaterial surfaces after implantation. The composition of the complex mixture of host factors that are deposited on the implant surface changes as the implant ages. Fibrinogen is a major component of newly implanted biomaterial and is the dominant ligand promoting *S. aureus* adherence. After longer exposure, deposited fibrinonectin becomes the dominant ligand. *S. aureus* can adhere to many other host plasma and matrix proteins, such as vitronectin, collagen, laminin, and von Willebrand factor, but the relevance of these interactions to device-related infections is unclear. Invasive strains of staphylococci have been reported to bind more fibronectin than commensal strains can do. Although *S. aureus* binding to collagen has received considerable attention in recent years as a potentially important mechanism to trigger osteomyelitis, septic arthritis, or native valve endocarditis, no significant association between these infections and the presence of indwelling devices has been presented so far. The molecular characterization of several genes encoding *S. aureus* adhesins, namely two fibrinogen-binding proteins called ClfA (also named clumping factor) and ClfB, one collagen adhesion, one elastin-binding protein, and two distinct but related fibronectin-binding proteins, has been achieved. Site-specific mutants of *S. aureus* specifically defective in adhesion to fibrinogen, fibronectin, or collagen have been described and used in various *in vitro* and *in vivo* studies. In contrast, *S. aureus*, *S. epidermidis*, and other clinically relevant species of coagulase-negative staphylococci exhibit weaker interactions with host proteins. Molecular studies also indicate that coagulase- negative staphylococci express a more limited repertoire of adhesins. Recent reports suggest the presence in coagulase-negative staphylococci of a distant homologue of the clfA gene of *S. aureus* as a putative fibrinogen-binding surface protein called Fbe, and two non-homologous types of surface proteins combining adhesive and autolytic properties. One of them allows *S. epidermidis* to interact with human vitronectin, while the other one expresses fibronectin-binding activity in *S. saprophyticus*.

The production of pyogenic exudates or abscesses by *S. aureus* infections indicates a major role for neutrophils in the host defense against such infections. Another important aspect of the host response to staphylococcal infection is the directed migration of the phagocytes toward an infectious focus, which is acting as a stimulus. Establishment of an inflammatory focus leads to the liberation of humoral mediators, which attract and modulate the cellular components of the inflammatory response. This complex set of events involves activation or inactivation of several humoral pathways, which interact with the migrating phagocytes.

Mostly *S. epidermidis*, but sometimes also mixed cultures, have been isolated from colonized catheters recovered from patients. In addition, infection of catheters has been shown by scanning electron microscopy to demonstrate the mode of adhesion (Peters *et al*., 1981). Bacterial cells, primarily those of

staphylococci, followed by *Acinetobacter calcoaceticus* and *P. aeruginosa*, were shown to be attached to the inner surface of the catheter. The thickest bacterial layers were found in catheters infected by coagulase-negative staphylococci (CoNS). The bacteria appeared to be closely packed and cemented together by a slimy matrix. Biofilms have been demonstrated on nearly all kinds of catheters. In addition to *S. epidermidis*, *Staphylococcus aureus* and *Candida parapsilosis* are able to form a biofilm, which indicates that bacteria and yeasts colonize intravascular catheters by an adherent biofilm mode of growth. Both *S. aureus* and *Enterococcus faecalis* have been isolated from biofilm; occasionally, fungal cells or *Proteus mirabilis* have been identified. During the course of polymer colonization, staphylococcal cells produce large amounts of extracellular slime in which the cells are embedded and finally completely covered. The slime substance is not a true capsule, but is loosely bound to the staphylococcal cells.

Production of slime is necessary for *S. epidermidis* colonization and is also observed with many other pathogens, including *S. aureus*. The chemical composition of slime has been characterized for *S. aureus*, as well as *S. epidermidis.* Mack *et al*. (1996) purified a specific polysaccharide antigen of the biofilm-producing strains of *S. epidermidis* and were able to distinguish two polysaccharide fractions. The structure of this polysaccharide is unique and, according to its function of cellular aggregation, it is referred to as polysaccharide intercellular adhesion (PIA). Infections involving coagulase-negative staphylococci lead to a higher mortality rate (30.5%), compared with those without coagulase-negative staphylococci bacteria (16.9%). The virulence of the commensal *S. epidermidis* is a result of the foreign-body implant, which acts to inhibit the normal host defense. The biofilm reduces the effectiveness of antibiotic therapy, leaving explantation of infected devices as often the only available treatment. Therefore, the aim of much research has been directed at elucidating the mechanisms of bacterial adhesion and colonization of the device biomaterials. The reactivity of various biopolymers to interact with immunological or bacterial ligands and/or toxins has not been clarified.

## Effect on human effector cells

Our investigations were addressing the following questions:

1. The properties of the various bioprosthetic devices on their own to interact with human PBMCs as well as human neutrophils. For this purpose, PBMC after their isolation and purification were also stimulated with staphylococcal enterotoxin B (SEB), phorbol-12,13-myristate-acetate (PMA), or with sodiumfluoride (NaF). PMNs after interaction with bioprosthetic devices were stimulated with the bacterial peptide fMLP, PMA, as well as NaF. These agonists stimulate the target cells via different mode of actions (MHCII-TCR, heterotrimeric G-proteins, PKC, as well as low molecular weight G-proteins).
2. The stimulatory and down-regulatory properties of bioprosthetic devices coated with SEB, fMLP, LPS, $LTB_4$, C5a, IL-8, GRO-α, ENA-78, GCP-2, NAP2. The effects and cell surface markers, as well as inflammatory mediator release, were studied.
3. The release of various cytokines (e.g., IL-10, IL-12, TNF-α), as well as inflammatory mediators (e.g., $LTB_4$, $PGE_2$), enzyme release (e.g., elastase).
4. The expression of essential molecules in innate immunity (e.g., TLR2, 4-9), chemokine receptors (CXCR1, CXCR2), fMLP receptors, the components of the NADPH oxydase ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$), as well as enzymes for lipid mediator induction and metabolism (e.g., cyclooxygenase 2 and receptors for prostaglandins EP1-4). As an example are shown the effects of several bacterial as well as immunological ligands (fMLP, SEB, LPS).

Four different bioprosthetic devices were used in our studies: (i) silver collagen polyester, (ii) collagen polyester, (iii) prosthetic device ePTFE, (iv) prosthetic device ePTFE gelatine. The bioprosthetic devices were equally sized (0.5 x 0.5 cm) and cut under sterile conditions. They were used plain or precoated with various ligands. Subsequently, they were dried by air and used if needed. In additional experiments bioprosthetic devices were also incubated with either human endothelial cells or human fibroblasts. The supernatant of the various activated cells was assessed for IL-8, TNF-α, IL-10, IL-12, $PGE_2$ release. Cell surface expression of various receptors were studied by FACS analysis. It was shown that the various devices coated with different ligands affect IL-10 release (Figure 54.9A&B) significantly. Similarly, elastase release from human neutrophils occurs, depending on the device and the appropriate ligand (Figure 54.10A&B).

Our data support the following results:

1. The four different prosthetic devices interact differently with regard to the induction of cytokines.
2. Bioprosthetic devices precoated with various ligands exert differences with regard to cytokine release.
3. The expression of Toll-like or chemokine receptors or chemotaxin receptors on the effector cells differs depending on the nature of the bioprosthetic device and its ligand.
4. The various microbial products interact differently with regard to the nature of the bioprosthetic devices.

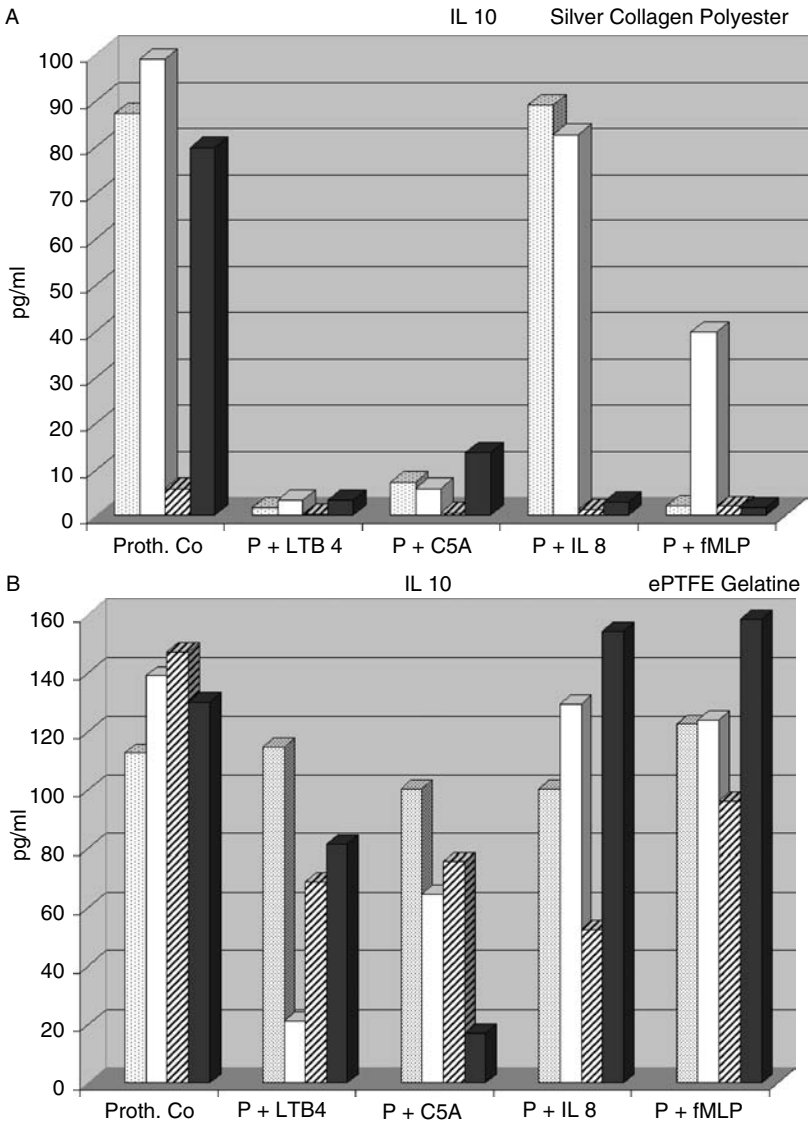



**FIGURE 54.9** IL-10 release from human peripheral mononuclear cells (PBMC).
PBMC ($1\times10^6$) were preincubated with bioprosthetic (P) devices, coated with $LTB_4$ (100 ng/ml), C5a (100 ng/ml), IL-8 (10 ng/ml), fMLP (0.1 µmol/ml) for 24 hours. Subsequently, they were stimulated with buffer (▨), SEB (□ 10 ng/ml); PMA (▨ 25 ng/ml); NaF (■ 5 nM) for further 24 hours. IL-10 was analyzed from the supernatant. Bioprosthetic devices: **A** – silver collagen polyester, **B** – ePTFE gelatine.

The results thus indicate that microbial toxins and exoproducts will bind to biopolymers and thus are responsible for induction of aseptic and most likely septic inflammation by facilitating the adherence of various bacteria. A more detailed analysis will contribute to our understanding of the role of bacterial protein toxins and/or microbial exoproducts when discussing immunopathological reactions induced by alloplastic devices.

## CONCLUSION

Our current knowledge of the strategies used by bacteria to interfere with innate and adaptive immunity and escape host defenses is largely incomplete. Nevertheless, the diverse disciplines in immunology, microbiology, infectious diseases, and cell biology have contributed much to the exciting progress we have made over recent years (Iwasaki and Medzhitov, 2004; Kanaoka and Boyce, 2004; Kaufmann *et al*., 2004; Schaffner *et al*., 2004; Stock *et al*., 2004; Yao *et al*., 1999). Today, sophisticated biochemical and genetic tools are being used to elucidate the molecular details surrounding host-pathogen interactions. These studies continue to provide detailed information on basic cellular mechanisms and offer novel avenues of potential chemotherapeutic intervention and even the possibility of introducing beneficial agents into eukaryotic cells. However, bacterial proteins including toxins can enter the cells, they can integrate themselves, and sometimes they abuse eukaryotic signaling mechanisms or deviate the immune response by their mimicry (Müllbacher *et al*., 2004; Nagata, 1997; Ruckdeschel *et al*., 2004).

Although a wide range of microbe-host relation ships can ultimately lead to disease, the two most general strategies used by pathogenic microbes may be

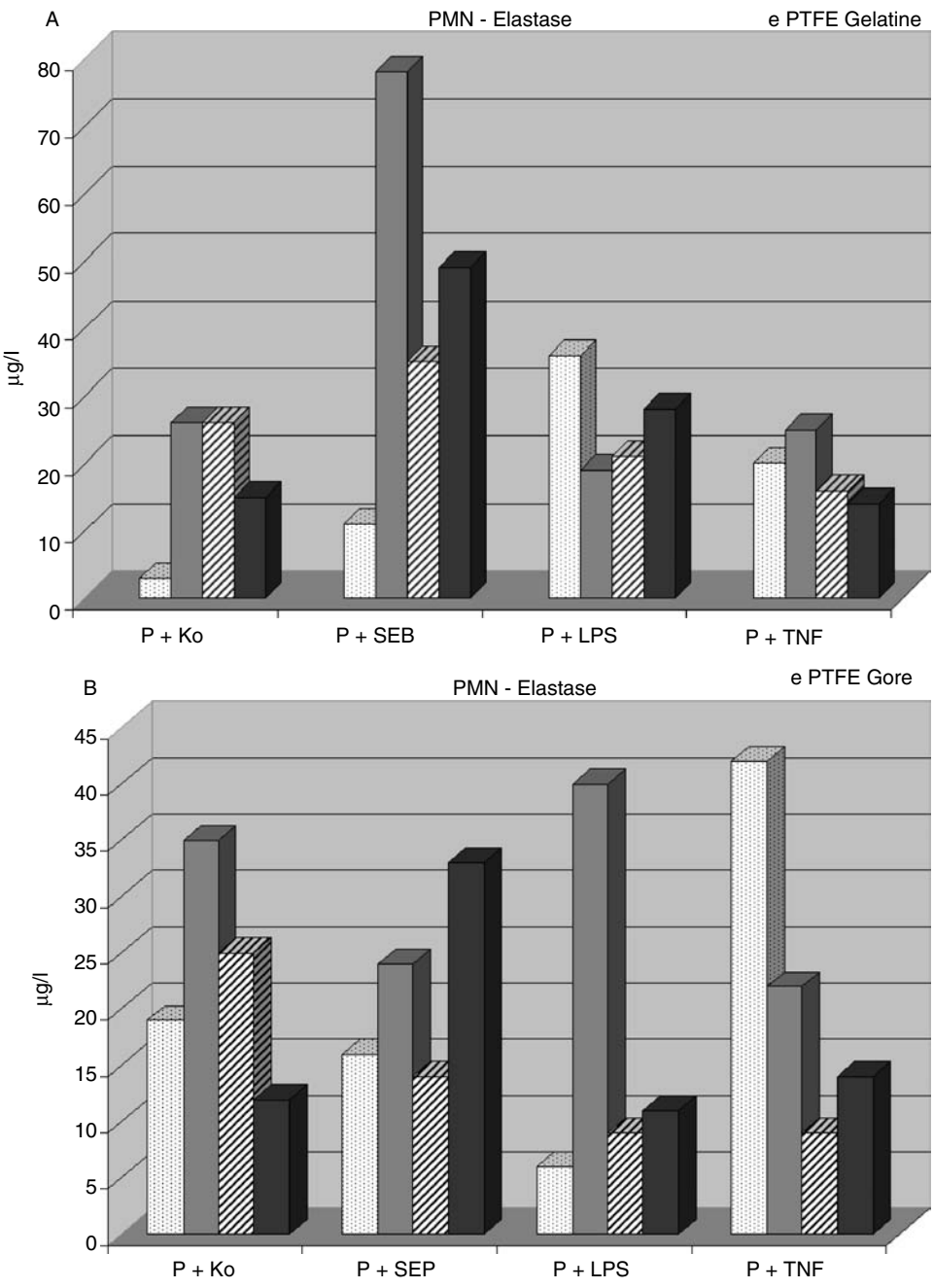



**FIGURE 54.10** Elastase release from human neutrophils in the presence of bioprosthetic devices (P) coated with various ligands SEB (10 ng/ml), LPS (500 pg/ml), TNF (100 U/ml). Neutrophils ($1\times10^6$/ml) were preincubated for 20 minutes and subsequently incubated with phosphate buffer (▨), fMLP (■);0.05 µmol/ml), PMA (▨); 25 ng/ml), NaF (■); 10 nM).
Bioprosthetic devices: **A** – ePTFE gelatine, **B** – ePTFE gore. Elastase release was analyzed from the cell supernatant.

described in military terms as "frontal" and "stealth" assaults. Pathogenic microorganisms make use of both of these approaches. Typically, frontal assault strategies require that the infecting microbes rapidly replicate, induce disease symptoms that overwhelm the innate defences of the host, and find a new host before engagement of the "adaptive" or "acquired" immune system, in which antigen-specific lymphocytes respond to antigen exposure. Stealth assaults, on the other hand, typically involve a slower infection process, in which the microbes subvert the host's innate and adaptive immune systems to set up a chronic or persistent infection. Bacterial protein toxins make use of both strategies.

Currently, we have to extend our views as to the effect of bacterial protein toxins by considering that toxins and other microbial exoproducts and/or microorganisms combined interact with host immune effector cells and furthermore that within bacterial biofilms toxins may exert persisting effects on the host immune response. The latter mechanisms are presently poorly understood and represent a future challenge in basic and clinical research.

## ACKNOWLEDGMENTS

The excellent secretarial support of Mrs. Kerstin Brennecke and Mrs. Marion Rentsch is greatly appreciated.

The current topics are supported by grants from the Deutsche Forschungsgemeinschaft (BA1671/3-1), Bundesministerium für Forschung und Technologie (BMBF: W. König -PP15: 01ZZ0107; B. König -PP17: 01ZZ0107, S. Backert -Start-up-project: 24)

## REFERENCES

Abman, S.H., Ogle, J.W., Butler-Simon, N., Rumack, C.M. and Accurso, F.J. (1988). Role of respiratory syncytial virus in early hospitalizations for respiratory distress of young infants with cystic fibrosis. *J. Pediatr*. **113,** 826–830.

Akira, S., Takeda, K. and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol*. **2,** 675–680.

Allen, L.A. (2003). Mechanisms of pathogenesis: evasion of killing by polymorphonuclear leukocytes. *Microbes Infect*. **5,** 1329–1335.

Allen, L.A. and Allgood, J. A. (2002). Atypical protein kinase C-zeta is essential for delayed phagocytosis of Helicobacter pylori. *Curr. Biol*. **12,** 1762–1766.

Allen, L.A., Schlesinger, L.S. and Kang, B. (2000). Virulent strains of *Helicobacter pylori* demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *J. Exp. Med*. **191,** 115–128.

Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J. and Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science* **300,** 1430–1434.

Amorim, C. Z., Hastewell, J. and Walker, C. H. (2002). Toll-like receptors as potential therapeutic targets for multiple diseases. *Nat. Rev. Drug Discovery* **1,** 797–807.

Aoki, M., Ono, Y., Kunii, O. and Goldstein, E. (1994). Effect of newer quinolones on the extra- and intracellular chemiluminescence response of human polymorpho-nuclear leucocytes. *J. Antimicrob. Chemother*. **34,** 383–390.

Arbour, N.C, Lorenz, E., Schutte, B.C, Zabner, J., Kline, J.N., Jones, M., Frees, K., Watt, J.L. and Schwartz, D.A. (2000). TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat. Genet*. **25,** 187–191.

Armstrong, D., Grimwood, K., Carlin, J.B., Carzino, R., Hull, J., Olinsky, A. and Phelan, P.D. (1998). Severe viral respiratory infections in infants with cystic fibrosis. *Pediatr. Pulmonol*. **26,** 371–379.

Arnold, R. and König, W. (2005). Respiratory syncytial virus infection of human lung endothelial cells enhances selectively ICAM-1 expression. *J. Immunol.* 174, 7359–7367.

Arnold, R. and König, W. (2005). Peroxisome proliferator-activated, receptor-gamma (PPAR-gamma) ligands possess antiviral and anti-inflammatory activity in the course of respiratory syncytial virus infection. *Allergy Clin. Immunol. Int.* (Suppl. 2), 2005. Hogrete and Huber Publishers.

Arnold, R., König, B., Werchau, H. and König, W. (2004). Respiratory syncytial virus deficient in soluble G protein induced an increased proinflammatory response in human lung epithelial cells. *Virology* **330,** 384–397.

Arnold, R., Scheffer, J., König, B. and König, W. (1993). Effect of *Listeria monocytogenes* and *Yersinia enterocolitica* on cytokine gene expression and release from polymorphonuclear granulocytes and human epithelial. *Infect. Immun*. **60,** 2545–2552.

Asada, K., Sasaki, S., Suda, T., Chida, K. and Nakamura, H. (2004) Anti-inflammatory roles of peroxisome proliferators—activated receptor γ in human alveolar macrophages. *Am. J. Respir. Crit. Care Med*. **169,** 195–200.

Backert, S., Churin, Y. and Meyer, T.F. (2002). *Helicobacter pylori* type IV secretion, host cell signaling, and vaccine development. *Keio J. Med*. **51** (Suppl. 2), 6–14.

Backert, S., Kwok, T., Schmid, M., Selbach, M., Moese, S., Peek, R.M.Jr., König, W., Meyer, T.F. and Jungblut, P.R. (2005). Subproteomes of soluble and structure-bound *Helicobacter pylori* proteins analyzed by two-dimensional gel electrophoresis and mass spectrometry *Proteomics*. 5, 1331–1345.

Backert, S., Moese, S., Selbach, M., Brinkmann, V. and Meyer, T.F. (2001). Phosphorylation of tyrosine 972 of the *Helicobacter pylori* CagA protein is essential for induction of a scattering phenotype in gastric epithelial cells. *Mol. Microbiol*. **42,** 631–644.

Backert, S., Schwarz, T., Miehlke, S., Kirsch, C., Sommer, C., Kwok, T., Gerhard, M., Goebel, U.B., Lehn, N., König, W. and Meyer, T.F. (2004). Functional analysis of the cag pathogenicity island in *Helicobacter pylori* isolates from patients with gastritis, peptic ulcer, and gastric cancer. *Infect. Immun*. **72,** 1043–1056.

Baggiolini, M. (2001). Chemokines in pathology and medicine. *J. Intern. Med*. **250,** 91–104.

Baggiolini, M. and Clark-Lewis, I. (1992). Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett*. **307,** 97–101.

Baker, M.D. and Acharya, K.R. (2004). Superantigens: Structure-function relationships. *Int. J. Med. Microbiol*. **293,** 529–537.

Barbieri, J.T., Riese, M.J. and Aktories, K. (2002). Bacterial toxins that modify the actin cytoskeleton. *Annu. Rev. Cell. Dev. Biol*. **18,** 315–344.

Becker, K., Friedrich, A.W., Lubritz, G., Weilert, M., Peters, G. and von Eiff, C. (2003). Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J. Clin. Microbiol*. **41,** 1434–1439.

Becker, M.N., Sauer, M.S., Muhlebach, M.S., Hirsh, A.J., Wu, Q., Verghese, M.W. and Randell, S.H. (2004). Cytokine secretion by cystic fibrosis airway epithelial cells. *Am. J. Respir. Crit. Care Med*. **169,** 645–653.

Berger, M., Budhu, S., Lu, E., Li, Y., Loike, D., Silverstein, S.C. and Loike, J.D. (2002). Different G(i)-coupled chemoattractant receptors signal qualitatively different functions in human neutrophils. *J. Leukoc. Biol*. **71,** 798–806.

Bergmann, U., Scheffer, J., Köller, M., Schönfeld, W., Erbs, G., Müller, F.E. and König, W. (1989). Induction of inflammatory mediators (histamine and leukotrienes) from rat peritoneal mast cells and human granulocytes by *Pseudomonas aeruginosa* strains from burn patients. *Infect. Immun*. **57,** 2187–2195.

Berin, M.C., Darfeuille-Michaud, A., Egan, L.J., Miyamoto, Y. and Kagnoff, M.F. (2002). Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. *Cell. Microbiol*. **4,** 635–648.

Beutler, B. (2004). Inferences, questions, and possibilities in Toll-like receptor signaling. *Nature* **430,** 257–263.

Bishop, A.L. and Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem. J.* **348,** 241–255.

Black, D.S. and Bliska, J.B. (2000). The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. *Mol. Microbiol*. **37,** 515–527.

Black, H.R., Yankaskas, J.R., Johnson, L.G. and Noah, T.L. (1998). IL-8 production by cystic fibrosis nasal epithelial cells after tumor necrosis factor alpha and respiratory syncytial virus stimulation. *Am. J. Respir. Cell. Mol. Biol*. **19,** 210–215.

Bodger, K. and Crabtree, J.E. (1998). Helicobacter pylori and gastric inflammation. *Br. Med. Bull*. **54,** 139–150.

Boncristiano, M., Paccani, S.R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., Amedei, A., D'Elios, M.M., Telford, J.L. and Baldari, C.T. (2003). The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J. Exp. Med*. **198,** 1887–1897.

Boone, D. L., Turer, E. E., Lee, E. G., Ahmad, R. C., Wheeler, M. T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O., McNally, E., Pickart, C. and Ma, A. (2004). The ubiquitin-modifying enzyme A20 is required for termination of *Toll-like* receptor responses. *Nat. Immunol*. **5,** 1052–1060.

Boquet, P. (2003). Lemichez E.Bacterial virulence factors targeting Rho GTPases: parasitism or symbiosis? *Trends Cell. Biol*. **13,** 238–246.

Bouchon, A., Facchetti, F., Weigand, M.A. and Colonna, M. (2001). TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* **410,** 1103–1107.

Boyle, E.C. and Finlay, B.B. (2003). Bacterial pathogenesis: exploiting cellular adherence. *Curr. Opin. Cell. Biol*. **15,** 633–639.

Brandt, S., Kwok, T., Hartig, R., König, W. and Backert, S. (2004). Potentiation of pro-inflammatory responses by the *Helicobacter pylori* CagA protein. Submitted.

Brandtzaeg, P. and Pabst, R. (2004). Let's go mucosal: communication on slippery ground. *Trends Immunol*. **25,** 570–577.

Brom, C., Brom, J. and König, W. (1992). GTPases and low molecular weight G-proteins during cell-cell interaction between neutrophils and platelets. *Int. Arch. All. Appl. Immunol*. **99,** 397–399.

Brom, J. and König, W. (1992). Cytokine-induced (IL-3, IL-6, IL-8, and TNF-β) activation and deactivation of human neutrophils. *Immunology* **75,** 281–285.

Brom, J., Köller, M., Müller-Lange, P.M., Steinau, H.U. and König, W. (1993). GTP-binding proteins in polymorphonuclear granulocytes of severely burned patients. *J. Leukoc. Biol*. **53,** 268–272.

Burns, D.L., Barbieri, J.T., Iglewski, B.H. and Rapppuoli, R. (2003). *Bacterial Protein Toxins*. ASM Press, Washington, DC.

Campbell, D.E. and Kemp, A.S. (1997). Proliferation and production of interferon-gamma (IFN-gamma) and IL-4 in response to *Staphylococcus aureus* and *sta*phylococcal superantigen in childhood atopic dermatitis. *Clin. Exp. Immunol*. **107,** 392–397.

Cannon, C.L., Kowalski, M.P., Stopak, K.S. and Pier, G.B. (2003). Pseudomonas aeruginosa—induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. *Am. J. Respir. Cell. Mol. Biol*. **29,** 188–197.

Chambers, H.F. (2001) The changing epidemiology of *Staphylococcus aureus*? *Emerg. Infect*. **7,** 178–182.

Chen, L.F. and Greene, W.C. (2004). Shaping the nuclear action of NF-kappaB. *Nat. Rev. Mol. Cell. Biol*. **5,** 392–401.

Chimini, G. and Chavrier, P. (2000). Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. *Nat. Cell. Biol*. **2,** E191–6.

Chmiel, J.F., Berger, M. and Konstan, M.W. (2002). The role of inflammation in the pathophysiology of CF lung disease. *Clin. Rev. in Allergy and Immunol*. **23,** 5–27.

Chow, C.W. Abreu, M.T.H., Suzuki, T. and Downey, G.P. (2003). Oxidative stress and acute lung injury. *Am. J. Respir. Cell. Mol. Biol*. **29,** 427–431.

Cook, D.N., Psietsky, D.S. and Schwartz, D.A. (2004). Toll-like receptors in the pathogenesis of human disease. *Nat. Immunol*. **5,** 975–979.

Cossart, P. and Sansonetti, P.J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* **304,** 242–248.

Covacci, A., Telford, J.L., Del Giudice, G., Parsonnet, J. and Rappuoli, R. (1999). Helicobacter pylori virulence and genetic geography. *Science* **284,** 1328–1333.

Cross, M.J. and Welsh, L.C. (2001). FGF and VEGF function in angiogenesis: signaling pathways, biological responses, and therapeutic inhibition. *Trends Pharmacol. Sci*. **22,** 201–207.

Dai, Y., Dean, T.P., Church, M.K., Warner, J.O. and Shute, J.K. (1994). Desensitisation of neutrophil responses by systemic interleukin 8 in cystic fibrosis. *Thorax* **49,** 867–871.

Dalton, J.E., Howell, G., Pearson, J., Scott, P. and Carding, S.R. (2004). Fas-Fas Ligand interactions are essential for the binding to and killing of activated macrophages by γδ T Cells. *J. Immunol*. **173,** 3660–3667.

Dayer, P.F., Schlegel-Haueter, S.E., Belli, D.C., Rochat, T., Dudez, T.S. and Suter, S. (1998). Cystic fibrosis and the pseudomonads. *J. Infect. Dis*. **177,** 1413–1417.

De Gregorio, E. and Rappuoli, R. (2004). Inside sensors detecting outside pathogens. *Nat. Immunol*. **5,** 1099–1100.

De Haan, L. and Hirst, T.R. (2004). Cholera toxin: a paradigm for multi functional engagement of cellular mechanisms (Review). *Mol. Membr. Biol*. **21,** 77–92.

Deng, J.C., Zeng, X., Newstead, M., Moore, T.A., Tsai, W.C., Thannickal, V.J. and Standiford, T.J. (2004). STAT4 is a critical mediator of early innate immune responses against pulmonary *K*lebsiella infection. *J. Immunol*. **173,** 4075–4083.

Devaney, J.M., Greene, C.M., Taggart, C.C., Carroll, T.P., O'Neill, S.J. and McElvaney, N.G. (2003). Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Lett*. **544,** 129–132.

Devine, D.A. (2003) Antimicrobial peptides in defense of the oral and respiratory tracts. *Mol. Immunol*. **40,** 431–443.

Diamond, G., Legarda, D. and Ryan, L.K. (2000). The innate immune response of the respiratory epithelium. *Immunol. Rev*. **173,** 27–38.

DiMango, E., Ratner, A.J., Bryan, R., Tabibi, S. and Prince, A. (1998a). Activation of NF-$_K$B by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. *J. Clin. Invest*. **101,** 2598–2605.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000). Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev*. **13,** 16–34.

Dragneva, Y., Anuradha, C.D., Valeva, A., Hoffmann, A., Bhakdi, S. and Husmann, M. (2001). Subcytocidal attack by staphylococcal alpha-toxin activates NF-kappaB and induces interleukin-8 production. *Infect. Immun*. **69,** 2630–3635.

Drynda, A., König, B., Bonventre, P.F. and König, W. (1995). Role of a carboxy-terminal site of toxic shock syndrome toxin 1 in eliciting immune responses of human peripheral blood mononuclear cells. *Infect. Immun*. **63,** 1095–1101.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D. and Vande Woude, G.F. (1998). Proteolytic inactivation of MAP kinase kinase by anthrax lethal factor. *Science* **280,** 734–737.

Dunzendorfer, S., Lee, H.K., Soldau, K. and Tobias, P.S. (2004). TLR4 is the signaling but not the lipopolysaccharide uptake receptor. *J. Immunol*. **173,** 1166–1170.

Enarsson, K., Innocenti-Brisslert, M., Backert, S. and Quiding-Järbrink, M. (2005). Helicobacter pylori induce transendothelial migration of activated memory T cells. *Infect. Immun*. **73,** 761–769.

Enright, M.C., Day, N.P., Davies, C.E., Peacock, S.J. and Spratt, B.G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol*. **38,** 1008–1015.

Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H. and Spratt, B.G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc. Natl. Acad. Sci. USA*. **99,** 7687–7692.

Epelman, S., Stack, D., Bell, C., Wong, E., Neely, G.G., Krutzik, S., Miyake, K., Kubes, P., Zbytnui, L.D., Ma, L.L., Xie, X., Woods, D.E. and Mody, CH. (2004). Different domains of *Pseudomonas*

*aeruginosa* exoenzyme S activate distinct TLRs. *J. Immunol*. **173,** 2031–2040.

Eriksson, S., Bjorkman, J., Borg, S., Syk, A., Pettersson, S., Andersson, D.I. and Rhen, M. (2000). Salmonella typhimurium mutants that downregulate phagocyte nitric oxide production. *Cell. Microbiol*. **2,** 239–250.

Esmon, C.T. (2004). Interactions between the innate immune and blood coagulation systems. *Trends Immunol*. **25,** 536–542.

Falnes, P.O. and Sandvig, K. (2000). Penetration of protein toxins into cells. *Curr. Opin. Cell. Biol*. **12,** 407–413.

Fasano, A. and Nataro, J.P. (2004). Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins. *Adv. Drug. Deliv. Rev*. **56,** 795–807.

Finlay, B.B. and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev*. **61,** 136–169.

Firoved, A.M., Ornatowski, W. and Deretic, V. (2004). Microarray analysis reveals induction of lipoprotein genes in mucoid Pseudomonas aeruginosa: implications for inflammation in cystic fibrosis. *Infect. Immun*. **72,** 5012–5018.

Fischer, S. and Adam, D. (2001). Effects of moxifloxacin on neutrophil phagocytosis, burst production, and killing as determined by a whole-blood cytofluorometric Method. *Antimicrob. Agents Chemother*. **45,** 2668–2669.

Frandji, P., Tkaczyk, C., Oskeritzian, C., David, B., Desaymard, C. and Mécheri, S. (1996). Exogenous and endogenous antigens are differentially presented by mast cells to CD4+ T lymphocytes. *Eur. J. Immunol*. **26,** 2517–2528.

Galan, J.E. and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284,** 1322–1328.

Galli, S.J. (1997). The Paul Kallos Memorial Lecture. The mast cell: a versatile effector cell for a challenging world. *Int. Arch. Allergy Immunol*. **113,** 14–22.

Gao, L.Y., Kwaik, Y.A. (2000). The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends Microbiol*. **8,** 306–313.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R. and Haas, R. (2003). Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301,** 1099–1102.

Gemmell, C.G. (1993). Antibiotics and neutrophil function—potential immunomodulating activities. *J. Antimicrob. Chemother*. **31** (Suppl.), B 23–33

Ghebremedhin, B., König, W. and König, B. (2005). Heterogeneity of methicillin-resistant Staphylococcus aureus strains at a German University Hospital during a one year period. *Eur. J. Clin. Microbiol. Infect. Dis*. **24**, 388–398.

Gieseler, S., König, B., König, W. and Backert, S. (2005). Strain-specific expression profiles of virulence genes in Helicobacter pylori during infection of gastric epithelial cells and granulocytes 7, 437–447.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., Coyle, A.J., DiStefano, P.S., Bertin, J., Sansonetti, P.J. and Philpott, D.J. (2003). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* **300,** 1584–1587.

Gobert, A.P., McGee, D.J., Akhtar, M., Mendz, G.L., Newton, J.C., Cheng, Y., Mobley, H. L. and Wilson, K.T. (2001). Helicobacter pylori arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc. Natl. Acad. Sci. USA*. **98,** 13844–13849.

Gouaux, E., Hobaugh, M. and Song, L. (1997). Alpha-hemolysin, gamma-hemolysin, and leukocidin from *Staphylococcus aureus:* distant in sequence but similar in structure. *Protein Sci*. **6,** 2631–2635.

Griese, M., Ramakers, J., Krasselt, A., Starosta, V., von Koningsbruggen, S., Fischer, R., Ratjen, F., Müllinger, B., Huber, R.M., Maier, K., Rietschel, E. and Scheuch, G. (2004). Improvement of alveolar glutathione and lung function but not oxidative state in cystic fibrosis. *Am. J. Respir Crit. Care. Med*. **169,** 822–828.

Gröner, M., Scheffer, J. and König, W. (1992). Modulation of leukotriene generation by invasive bacteria. *Immunology* **77,** 400–407.

Gutierrez, O., Pipaon, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, F., Nunez, G. and Fernandez-Luna, J.L. (2002). Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J. Biol. Chem*. **277,** 41701–41705.

Hadjivassiliou, M., Williamson, C.A. and Woodroofe, N. (2004). The immunology of gluten sensitivity: beyond the gut. *Trends Immunol*. **25,** 578–582.

Haehnel, V., Schwarzfischer, L., Fenton, M.J. and Rehli, M. (2002). Transcriptional regulation of the human toll-like receptor 2 gene in monocytes and macrophages. *J. Immunol*. **168,** 5629–5637.

Hajjar, A.M., Ernst, R.K., Tsai, J.H., Wilson, C.B. and Miller, S.I. (2002). Human toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol*. **3,** 354–359.

Hashimoto, S., Kobayashi, A., Kooguchi, K., Kitamura, Y., Onodera, H. and Nakajima, H. (2000). Upregulation of two death pathways of perforin/granzyme and FasL/Fas in septic acute respiratory Distress Syndrome. *Am. J. Respir. Crit. Care. Med*. **161,** 237–243.

Hauf, N. and Chakraborty, T. (2003). Suppression of NF-kappa B activation and pro-inflammatory cytokine expression by Shiga toxin-producing Escherichia coli. *J. Immunol*. **170,** 2074–2082.

Henderson, B., Poole, S. and Wilson, M. (1996). Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev*. **60,** 316–341.

Hensler, T., Köller, M., Alouf, J.E. and König, W. (1993a). Toxic Shock Syndrome toxin 1 and the erythrogenic toxin A modulate the chemotactic response of human neutrophils. *Infect. Immun*. **58,** 1055–1061.

Hensler, T., Köller, M., Prévost, G., Piémont, Y. and König, W. (1994b). GTP-binding proteins are involved in the modulated activity of human neutrophils treated with the Panton-Valentine leukocidin from Staphylococcus aureus. *Infect. Immun*. **62,** 5281–5289.

Hensler, T., König, B., Prévost, G., Piémont, Y., Köller, M. and König, W. (1994c). Leukotriene B4-generation and DNA fragmentation induced by leukocidin from Staphylococcus aureus: protective role of granulocyte-macrophage colony-stimulating factor (GMCSF) and G-CSF on human neutrophils. *Infect. Immun*. **62,** 2529–2535.

Hensler, T., Köller, M., Alouf, J.E. and König, W. (1991). Bacterial toxins induce heat shock proteins in human neutrophils. *Biochem. Biophys. Res. Commun*. **179,** 872–879.

Hiatt, P.W., Grace, S.C., Kozinetz, C.A., Raboudi, S.H., Treece, D.G., Taber, L.H. and Piedra, P.A. (1999). Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. *Pediatrics* **103,** 619–626.

Hoebe, K., Janssen, E. and Beutler, B. (2004). The Interface between innate and adaptive immunity. *Nat. Immunol*. **5,** 971–974.

Hofer, M.F., Lester, M.R., Schlievert, P.M. and Leung, D.Y. (1995). Upregulation of IgE synthesis by staphylococcal toxic shoch syndrome toxin-1 in peripheral blood mononuclear cells from patients with atopic dermatitis. *Clin. Exp. Allergy*. **25,** 1218–1227.

Hollenbach, E., Neumann, M., Vieth, M., Roessner, A., Malfertheiner, P. and Naumann, M. (2004). Inhibition of p38 MAP kinase- and RICK/NF-kB-signaling suppresses inflammatory bowel disease. *Faseb J*. **18,** 1550–1552.

Holst, P.J. (2003). Rosenkilde M. M.Microbiological exploitation of the chemokine system. *Microbes Infect*. **5,** 179–187.

Hornef, M.W., Wick, M.J., Rhen, M. and Normark, S. (2002). Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol*. **3,** 1033–1040.

Horng, T., Barton, G.M., Flavell, R.A. and Medzhitov, R. (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420,** 329–333.

Hoshino, K., Takeuchi, O., Kawai, T.,Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol*. **162,** 3749–3752.

Howard, A.D., McAllister, G., Feighner, S.D., Liu, Q., Nargund, R.P., Van der Ploeg, L.H.T. and Patchett, A.A. (2001). Orphan G-protein-coupled receptors and natural ligand discovery. *Trends Pharmakol. Sci*. **22,** 132–140.

Ishihara, S., Rumi, M.A.K., Kadowaki, Y., Ortega-Cava, C.F., Yuki, T., Yoshino, N., Miyaoka, Y., Kazumori, H., Ishimura, N., Amano, Y. and Kinoshita, Y. (2004). Essential role of MD-2 in TLR4-dependent signaling during Helicobacter pylori-associate gastritis. *J. Immunol*. **173,** 1406–1416.

Iwasaki, A. and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nat. Immunol*. **5,** 987–995.

Iyer, S.S., Barton, J.A., Bourgoin, S. and Kusner, D.J. (2004) Phospholipases D1 and D2 coordinately regulate macrophage phagocytosis. *J. Immunol*. **173,** 2615–2623.

Jaeger, K. E., Kinscher, D. A., König, B. and König, W. (1992). Extracellular lipase of pseudomonas aeruginosa: Biochemistry and potential role as a virulence factor. In: *European Congress of Cystic Fibrosis* (eds. Pedersen and Hoiby), pp. 113–119.

Janeway, C.A, Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. *Cold. Spring. Harb. Symp. Quant. Biol*. **54,** 1–13.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meunier, H., Forey, F., Nesme, X., Etienne, J. and Vandenesch, F. (2002). Relationships between Staphylococcus aureus genetic background, virulence factors, agr type (alleles), and human disease type. *Infect. Immun*. **70,** 631–641.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., Bonneville, M. and Lina, G. (2001). Phylogenetic analysis of enterotoxin genes indicated that they all potentially derived from this cluster, identifying egc as a putative nursery of enterotoxin genes. *J. Immunol*. **166,** 669–677.

Ji, G., Beavis, R. and Novick, R.P. (1997) Bacterial interference caused by autoinducing peptide variants. *Science* **276,** 2027–2030.

Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375,** 500–503.

Kagnoff, M.F. and Eckmann, L. (1997). Epithelial cells as sensors for microbial infection. *J. Clin. Invest*. **100,** 6–10.

Kahl, B.C., Becker, K., Friedrich, A.W., Clasen, J., Bhanu, S., von Eiff, C. and Peters, G. (2003a). Agr-dependent bacterial interference has no impact on long-term colonization of Staphylococcus aureus during persistent airway infection of cystic fibrosis patients. *J. Clin. Microbiol*. **41,** 5199–5201.

Kahl, B.C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R.A. and Peters, G. (2003b). Thymidine-dependent small-colony variants of Staphylococcus aureus exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. *J. Clin. Microbiol*. **41,** 410–413.

Kanaoka, Y. and Boyce, J.A. (2004). Cysteinyl leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses. *J. Immunol*. **173,** 1503–1510.

Kao, C.Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R.W. and Wu, R. (2004). IL-17 markedly up-regulates β-defensin-2 expression in human airway epithelium via JAK and NF-kB signaling pathways. *J. Immunol*. **173,** 3482–3491.

Kaufmann, S. H. E., Medzhitov, R. and Gordon, S. (2004). *The Innate Immune Response to Infection*. ASM Press, Washington, DC.

Kawei, T., Sato, S., Ishii, K.J., Coban, C., Hemmi, H., Yamamoto, M., Terai, K., Matsuda, M., Inoue, J-i., Uematsu, S., Takeuchi, O. and Akira, S. (2004). Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat. Immunol*. **5,** 1061–1068.

Kayal, S., Lilienbaum, A., Join-Lambert, O., Li, X., Israel, A. and Berche, P. (2002). Listeriolysin O secreted by Listeria monocytogenes induces NF-kappaB signaling by activating the IkappaB kinase complex. *Mol. Microbiol*. **44,** 1407–19.

Kelsall, B.L. and Rescigno, M. (2004). Mucosal dendritic cells in immunity and inflammation. *Nat. Immunol*. **5,** 1091–1095.

Kerr, J.R. (1999). Cell adhesion molecules in the pathogenesis of and host defense against microbial infection. *Mol. Pathol*. **52,** 220–230.

Kimata, H., Fujimoto, M., Ishioka, C. and Yoshida, A. (1996). Histamine selectively enhances human immunoglobulin E (iGE) and IgG4 production induced by anti-CD58 monoclonal antibody. *J. Ex. Med*. **184,** 357–364.

Knodler, L.A, Celli, J. and Finlay, B.B. (2001). Pathogenic trickery: deception of host cell processes. *Nat. Rev. Mol. Cell. Biol*. **2,** 578–88.

Köller, M., Brom, J. and König, W. (1993). Analysis of 5-lipoxygenase and 5-lipoxygenase activating protein in neutrophil granulocytes of severely burned patients. In: *Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury*. (Eds. Nigam, S., Honn, C. W., Walden, T. Jr., Kluwer), pp. 759–761. Academic Press, Boston.

König, B. and König, W. (1991). Role of protein kinase C and G-proteins in human peripheral blood leukocytes on inflammatory mediator release by genetically cloned E. coli strains (hemolysin +/−; adhesins +/−). *Infect. Immun*. **59,** 3801–3810.

König, B. and König, W. (1993). Induction and suppression of cytokine release (tumor necrosis factor-alpha; interleukin-6; interleukin 1β) by Escherichia coli pathogenicity factors (adhesins, alpha-hemoylsin). *Immunology* **78,** 526–533.

König, B. and König, W. (2005). The fluoroquinolone Moxifloxacin modulates neutrophil chemotaxis. Submitted.

König, B., Jaeger, K.E., Sage, A.E., Vasil, M.L. and König, W. (1996a). Role of *Pseudomonas aeruginosa* lipase in inflammatory mediator release from human inflammatory effector cells (platelets, granulocytes, and monocytes). *Infect. Immun*. **64,** 3252–3258.

König, B., Köller, M., Prévost, G. *et al*. (1994a). Activation of effector cells by different bacterial toxins (leukocidin, alveolysin, erythrogenic toxin A), generation of interleukin-8. *Infect. Immun*. **62,** 4831–4837.

König, B., König, W., Arnold, R., Werchau, H., Ihorst, G. and Forster, J. (2004). Prospective study of human metapneumovirus infection in children less than three years of age. *J. Clin. Microbiol*. **42,** 4632–4635.

König, B., Ludwig, A., Goebel, W. and König, W. (1994). Pore formation by the Escherichia coli α-hemolysin: role for mediator release from human inflammatory cells. *Infect. Immun*. **63,** 4612–4617.

König, B., Prévost, G. and König, W. (1997). Composition of staphylococcal bi-component toxins determines pathophysiological reactions. *J. Med. Microbiol*. **46,** 479–485.

König, B., Prévost, G., Piémont, Y. and König, W. (1995). Effects of *Staphylococcus aureus* leukocidins on inflammatory mediator release from human granulocytes. *J. Infect. Dis*. **171,** 607–613.

König, B., Tammer, I., Sollich, V. and König, W. (2004). Intra- and interpatient variability of the hsp65 and 16S-23S intergenic gene region in Mycobacterium abscessus strains from Cystic Fibrosis patients. Submitted.

König, B., Vasil, M.L. and König, W. (1997). Role of hemolytic and non-hemolytic phospholipase C from Pseudomonas aeruginosa in intelreukin-8 release from human monocytes. *J. Med. Microbiol*. **46,** 471–478.

König, W., Fischer, U., Stephan, Th. and Bujanowski-Weber, J. (1991). Regulation of CD23 in allergic disease. In: *Monographs in*

*Allergy*—CD23, a novel multifunctional regulator of the immune system (ed. J. Gordon), pp. 94–123. Karger, Basel.

König, W., Köller, M. and Brom J. (1992). Priming mechanisms and induction of heat shock proteins in human polymorphonuclear granulocytes induced by eicosanoids and cytokines. *Eisosanoids* **5,** 539–541.

König, W., Schönfeld, W., Raulf, M., Köller, M., Knöller, J., Scheffer, J. and Brom, J. (1990). The neutrophil and leukotrienes' role in health and disease. *Eicosanoids* **3,** 1–22.

Kotone-Miyahara, Y., Yamashita, K., Lee, K., Yonehara, S., Uchiyama, T., Sasada, M. and Takashashi, A. (2004). Short-term delay of Fas-stimulated apoptosis by GM-CSF as a result of temporary suppression of FADD recruitment in neutrophils: evidence implicating phosphatidylinositol 3-kinase and MEK1/2 pathways downstream of classical protein kinase C. *J. Leukoc. Biol*. **76,** 1047–1056.

Kotwal, G.J. (1997). Microorganisms and their interaction with the immune system. *J. Leukoc. Biol*. **62,** 415–429.

Krell, S., Adams, I., Arnold, U., Kalinski, T., Aumann, V., König, W. and König, B. (2003). Influenza B pneumonia with Staphylococcus aureus superinfection associated with Parvovirus B19 and concomitant agranulocytosis. *Infection* **5,** 353–358.

Kunkel, S.L., Lukacs, N.W., Strieter, R.M. and Chensue, S.W. (1999). The role of chemokines in the immunopathology of pulmonary disease. *Forum* (Genova). **9,** 339–355. Review.

Kwok, T., Backert, S., Schwarz, H., Berger, J. and Meyer, T.F. (2002). Specific entry of Helicobacter pylori into cultured gastric epithelial cells via a zipper-like mechanism. *Infect. Immun*. **70,** 2108–2120.

Labro, M.T. (1993). Interaction of macrolides and quinolones with the host defense system. *Eur. Bull. Drug. Res*. **2** (Suppl. 1), 7–13.

Labro, M.T. (2000). Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales"? *Clin. Microbiol. Rev*. **13,** 615–650.

Lazarus, R., Raby, B.A., Lange, C., Silverman, E.K., Kwiatkowski, D.J., Vercelli, D., Klimecki, W.J., Martinez, F.D. and Weiss, S.T. (2004). Toll-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am. J. Respir. Crit. Care. Med*. **170,** 594–600.

Lee, S.K., Stack, A., Katzowitsch, E., Aizawa, S.I., Suerbaum, S. and Josenhans, C. (2003). Helicobacter pylori flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. *Microbes Infect*. **5,** 1345–1356.

Leung, D.Y. (1998). Molecular basis of allergic diseases. *Mol. Genet. Metab*. **63,** 157–167.

Levy, O. (2004). Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. *J. Leukoc. Biol*. **76,** 909–925.

Li, H., Llera, A. and Mariuzza, R.A. (1998). Structure-function studies of T cell receptor superantigen interactions. *Imm. Rev*. **163,** 177–186.

Li, Y., Li, H., Dimasi, N., McCormick, J.K., Martin, R., Schuck, P., Schlievert, P.M. and Mariuzza, R.A., (2001). Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity* **14,** 93–104.

Lin, T.J., Maher, L.H., Gomi, K., McCurdy, J.D., Garduno, R. and Marshall, J.S. (2003). Selective early production of CCL20, or macrophage inflammatory protein 3alpha, by human mast cells in response to Pseudomonas aeruginosa. *Infect. Immun*. **71,** 365–373.

Lina, G., Boutite, F., Tristan, A., Bes, M., Etienne, J. and Vandenesch, F. (2003). Bacterial competition for human nasal cavity colonization: role of Staphylococcal agr alleles. *Appl. Envir. Microbiol*. **69,** 18–23.

Lina, G., Piémont, Y., Godail-Gamot, F., Bes, M., Peter, M.O., Gauduchon, V., Vandenesch, F. and Etienne J. (1999). Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. *Clin. Infect. Dis*. **29,** 1128–1132.

Liston, A. and McColl, S. (2003). Subversion of the chemokine world by microbial pathogens. *Bioessays*. **25,** 478–88.

Loetscher, P. and Clark-Lewis, I. (2001). Agonistic and antagonistic activities of chemokines. *J. Leukoc. Biol*. **69,** 881–884.

Lorenz, E, Chemotti, D.C., Vandal, K. and Tessier, P.A. (2004). Toll-like receptor 2 represses nonpilus adhesin-induced signaling in acute infections with the Pseudomonas aeruginosa pilA mutant. *Infect. Immun*. **72,** 4561–4569.

Mack, D.M., Haeder, M., Siemssen, N. and Laufs, R. (1996). Association of biofilm production of coagulase-negative staphylococci with expression of a specific polysaccharide intercellular adhesin. *J. Inf. Dis*. **174,** 881–884.

Mackay, C.R. (2001). Chemokines: immunology's high impact factors. *Nat. Immunol*. **2,** 95–101.

MacMicking, J.D. (2004). IFN-inducible GTPases and immunity to intracellular pathogens. *Trends Immunol*. **25,** 601–609.

Maggi, E. (1998). The Th1/Th2 paradigm in allergy. *Immunotechnol*. **3,** 233–244.

Mahalingam, S. and Karupiah, G. (1999). Chemokines and chemokine receptors in infectious diseases. *Immunol. Cell. Biol*. **77,** 469–475.

Mantovani, A., Bussolino, F. and Introna, M. (1997). Cytokine regulation of endothelial cell function: from molecular level to the bedside. *Immunol. Today* **18,** 231–239.

Marinissen, M.J. and Gutkind, J.S. (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci*. **22,** 368–375.

Maus, U.A., Srivastava, M., Paton, J.C., Mack, M., Everhart, M.B., Blackwell, T.S., Christman, J.W., Schlönforff, D., Seeger, W. and Lohmeyer, J. (2004). Pneumolysin-induced lung injury is independent of leukocyte trafficking into the alveolar space. *J. Immunol*. **173,** 1307–1312.

McCormick, J.M., Yarwood, J.M. and Schlievert, P.M. (2001). Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol*. **55,** 77–104.

Mécheri, S. and David, B. (1997). Unravelling the mast cell dilemma: culprit or victim of its generosity? *Immunol. Today* **18,** 212–215.

Medzhitov, R. and Janeway, C., Jr. (2000). Innate immunity. *New Engl. J. Med*. **343,** 338–344.

Merrell, D.S. and Falkow, S. (2004). Frontal and stealth attack strategies in microbial pathogenesis. *Nature* **430,** 250–256.

Middleton, P.G., Pollard, K.A., Donohoo, E., Wheatley, J.R. and Geddes, D.M. (2003). Airway surface liquid calcium modulates chloride permeability in the cystic fibrosis airway. *Am. J. Respir. Crit. Care. Med*. **168,** 1223–1226.

Mimuro, H., Suzuki, T., Tanaka, J., Asahi, M., Haas, R. and Sasakawa, C. (2002). Grb2 is a key mediator of Helicobacter pylori CagA protein activities. *Mol. Cell*. **10,** 745–755.

Moese, S., Selbach, M., Meyer, T.F. and Backert, S. (2002). $cag^+$ Helicobacter pylori induces homotypic aggregation of macrophage-like cells by up-regulation and recruitment of intracellular adhesion molecule 1 to the cell surface. *Infect. Immun*. **70,** 4687–4691.

Monack, D.M., Mueller, A. and Falkow, S. (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat. Rev. Microbiol*. **2,** 747–765.

Montecucco, C. and Rappuoli, R. (2001). Living dangerously: how Helicobacter pylori survives in the human stomach. *Nat. Rev. Mol. Cell. Biol*. **2,** 457–466.

Montecucco, C., De Bernard, M., Papini, E. and Zoratti, M. (2001). Helicobacter pylori vacuolating cytotoxin: cell intoxication and anion-specific channel activity. *Curr. Top. Microbiol. Immunol*. **257,** 113–129.

Moser, C., Johansen, H.K., Song, Z., Hougen, H.P., Rygaard, J. and Hoiby, N. (1997). Chronic Pseudomonas aeruginosa lung infection is more severe in Th2 responding BALB/c mice compared to Th1 responding C3H/HeN mice. *APMIS* **105,** 838–842.

Muir, A., Soong, G., Sokol, S., Reddy, B, Gomez, M.I., Van Heeckeren, A. and Prince, A. (2004). Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am. J. Respir. Cell. Mol. Biol*. **30,** 777–783.

Müllbacher, A., Regner, M., Wang, Y., Lee, E., Lobigs, M. and Simon, M. (2004). Can we really learn from model pathogens? *Trends Immunol*. **25,** 524–28.

Müller, M., Stass, H., Brunner, M., Möller, J.G., Lackner, E. and Eichler, H.G. (1999). Penetration of moxifloxacin into peripheral compartments in humans. *Antimicrob. Agents Chemother*. **43,** 2345–2349.

Müller-Alouf, H., Alouf, J.E., Gerlach, D., Ozegowski, J.H., Fitting, C. and Cavaillon, J. M. (1996). Human pro- and anti-inflammatory cytokine patterns induced by Streptococcus pyogenes erythrogenic (pyrogenic) exotoxin A and C superantigens. *Infect. Immun*. **64,** 1450–1453.

Müller-Alouf, H., Gerlach, D., Desreumaux, P., Leportier, C., Alouf, J.E. and Capron, M. (1997). Streptococcal pyrogenic exotoxin A (SPE A) superantigen-induced production of hematopoietic cytokines, IL-12 and IL-13, by human peripheral blood mononuclear cells. *Microb. Pathog*. **23,** 265–272.

Murphy, G.J. and Holder, J.C. (2000). PPAR-γ agonists: therapeutic role in diabetes, inflammation and cancer. *TIPS* **21,** 469–474.

Muzio, M., Bosisio, D., Polentarutti, N., D'amico, G., Stoppacciaro, R. Mancinelli, C., van't Veer, G., Penton-Rol, L.P., Ruco, P. Allavena and Mantovani, A. (2000). Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol*. **164,** 5998–6004.

Nagata, S. (1997). Apoptosis by death factor. *Cell* **88,** 355–365.

Neuber, K. and König, W. (1992). Effects of Staphylococcus aureus cell wall products (teichoic acid, peptidoglycan) and enterotoxin B on immunoglobulin (IgE, IgA, IgE) synthesis and CD23 expression in patients with atopic dermatitis. *Immunology* **75,** 23–28.

Neuber, K., Hilger, R.A. and König, W. (1991a). Interleukin-3, Interleukin-8, FMLP, and C5a enhance the release of leukotrienes from neutrophils of patients with atopic dermatitis. *Immunology* **73,** 197–204.

Neuber, K., Hilger, R.A. and König, W. (1992). Differential Increase in 12-HETE release and CD29/CD49f expression of platelets from normal donors and from patients with atopic dermatitis by Staphylococcus aureus. *Int. Arch. All. Appl. Immunol*. **98,** 339–342.

Neuber, K., Stephan, U., Fränken, J. and König, W. (1991b). Staphylococcus aureus modifies the cytokine-induced immunoglobulin synthesis and CD23 expression in patients with atopic dermatitis. *Immunology* **73,** 197–204.

Nikolaidis, N.M, Zimmermann, N., King, N.E., Mishra, A., Pope, S.M., Finkelman, F.D. and Rothenberg, M.E. (2003). Trefoil Factor-2 is an allergen-induced gene regulated by Th2 cytokines and STAT6 in the lung. *Am. J. Respir. Cell. Mol. Biol*. **29,** 458–464.

Novick, R. P. (2000). Pathogenicity factors and their regulation. In: *Gram-Positive Pathogens* (eds. V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy and J. I. Rood), pp. 392–407. American Society for Microbiology, Washington, D.C.

Novick, R.P., Schlievert, P. and Ruzin, A. (2001). Pathogenicity islands of staphylococci. *Microbes Infect*. **3,** 585–594.

Oliveira, D.C. and de Lencastre, H. (2002). Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. *Antimicrob. Agents Chemother*. **36,** 2155–2161.

Omoe, K., Ishikawa, M., Shimoda, Y., Hu, D.L., Ueda, S. and Shinagawa, K. (2002). Detection of seg, seh, and sei genes in Staphylococcus aureus isolates and determination of the enterotoxin productivities of S aureus isolates harboring seg, seh, or sei genes. *J. Clin. Microbiol* **40,** 857–862.

Papageorgiou, A.C., Quinn, C.P., Beer, D., Brehm, R.D., Tranter, H.S. and Bonventre, P.F. (1996). Crystal structure of a biologically inactive mutant of toxic shock syndrome toxin-1 at 2.5 A resolution. *Protein Sci*. **5,** 1737–1741.

Park, H.S., Jung, H.Y., Kim, J., Lee, W.J. and Bae, Y.S. (2004). Cutting edge: Direct interaction of TLR 4 with NAD(P) H Oxidase 4 Isozyme is essential for Lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kB. *J. Immunol*. **173,** 3589–3593.

Peek, R.M., Jr., Blaser, M.J. (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer* **2,** 28–37.

Perez-Perez, G.I., Shepherd, V.L., Morrow, J.D. and Blaser M.J. (1995). Activation of human THP-1 cells and rat bone marrow-derived macrophages by Helicobacter pylori lipopolysaccharide. *Infect. Immun*. **63,** 1183–1187.

Peters, G., Locci, R. and Pulverer, G. (1981). Microbial colonization of prosthetic divices. II. Scanning electron microscopy of naturally infected intravenous catheters. *Zentralbl. Bakteriol. Micrbiol. Hyg. B*. **173,** 293–299.

Petersson, K., Forsberg, G. and Walse, B. (2004). Interplay between superantigens and immunoreceptors. *Scandinavian J. Immunol*. **59,** 345–355.

Petersson, K., Hakansson, M., Nilsson, H., Forsberg, G., Svensson, L.A., Liljas, A. and Walse, B. (2001). Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. *EMBO J*. **20,** 3306–3312.

Petersson, K., Pettersson, H., Skartved, N.J., Walse, B. and Forsberg, G. (2003). Staphylococcal enterotoxin H induces V alpha-specific expansion of T cells. *J. Immunol*. **170,** 4148–4154.

Pier, G.B., Grout, M. and Zaidi, T.S. (1997). Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung. *Proc. Natl. Acad. Sci. USA*. **94,** 12088–12093.

Power, M.R., Peng, Y., Maydanski, E., Marshall, J.S. and Lin, T.J. (2004). The development of early host response to Pseudomonas aeruginosa lung infection is critically dependent on MyD88 in mice. *J. Biol Chem*. (in press)

Prévost, G., Cribier, B., Couppié, P. *et al*. (1995). Panton-Valentine leucocidin and gamma-hemolysin from Staphylococcus aureus ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immun*. **63,** 4121–4129.

Quinn, M.T. and Gauss, K.A. (2004) Structure and regulation of the neutrophil respiratory burst oxidase: comparison with nonphagocyte oxidases. *J. Leukoc. Biol*. **76,** 760–781.

Ramarao, N., Gray-Owen, S.D., Backert, S. and Meyer, T. (2000). *Helicobacter pylori* inhibits phagocytosis by professional phagocytes involving type IV secretion components. *Mol. Microbiol*. **37,** 1389–1404.

Raveh, D., Kruskal, B.A., Farland, J. and Ezekowitz, R.A.B. (1998). Th1 and Th2 cytokines cooperate to stimulate mannose-receptor-mediated phagocytosis. *J. Leukoc Biol*. **64,** 108–113.

Re, F. and Strominger, J.L. (2001). Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem*. **276,** 37692–37699.

Riches, D.W. (2004). Peroxisome proliferator-active receptor γ: A legitimate target to control pulmonary inflammation? *Am. J. Respir. Crit. Care. Med*. **169,** 145–146.

Romani, L., Puccetti, P. and Bistoni, F. (1997). Interleukin-12 in infectious diseases. *Clin. Microbiol. Rev*. **10,** 611–636.

Ruckdeschel, K., Pfaffinger, G., Haase, R., Sing, A., Weighardt, H., Häcker, G., Holzmann, B. and Heesemann, J. (2004). Signaling of apoptosis through TLRs critically involves Toll/IL-1 receptor domain-containing adapter inducing IFN-β, but not MyD88, in Bacteria-Infected murine macrophages. *J. Immunol*. **173,** 3320–3328.

Sabroe, I., Jones, E.C., Usher, L.R., Whyte, K.B.M. and Dower, S.K. (2002). Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: A critical role for monocytes in leukocyte lipopolysaccharide responses. *J. Immunol*. **168,** 4701–4710.

Saloga, J., Leung, D.Y., Reardon, C., Giorno, R.C., Born, W. and Gelfand, E.W. (1996). Cutaneous exposure to the superantigen staphylococcal enterotoxin B elicits a T cell-dependent inflammatory response. *J. Invest. Dermatol*. **106,** 982–988.

Schaffner, A., King, C.C., Schaer, D. and Guiney, D.G. (2004). Induction and antimicrobial activity of platelet basic protein derivatives in human monocytes. *J. Leukoc. Biol*. **76,** 1010–1018.

Schiavo, G. and van der Goot, F.G. (2001). The bacterial toxin toolkit. *Nat. Rev. Mol. Cell. Biol*. **2,** 530–537.

Schmidt, K.D., Tümmler, B. and Römling, U. (1996). Com parative genome mapping of Pseudomonas aeruginosa PAO with P. aeruginosa C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. *J. Bacteriol*. **178,** 85–93.

Selbach, M., Moese, S., Backert, S., Jungblut, P.R. and Meyer, T.F. (2004). The Helicobacter pylori CagA protein induces tyrosine dephosphorylation of ezrin. *Proteomics* **4,** 2961–2968.

Selbach, M., Moese, S., Hurwitz, R., Hauck, C.R., Meyer, T.F. and Backert, S. (2003). The Helicobacter pylori CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO J*. **22,** 515–528.

Selbach, M., Moese, S., Meyer, T.F. and Backert, S. (2002). Functional analysis of the Helicobacter pylori cag pathogenicity island reveals both VirD4-CagA-dependent and VirD4-CagA-independent mechanisms. *Infect. Immun*. **70,** 665–671.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med*. **189,** 1777–1782.

Shortman, K. and Wu, L. (2004). Are dendritic cells and cells? *Nat. Immunol*. **5,** 1105–6.

Silverman, E.S. and Drazen, J.M. (2003). Immunostimulatory DNA for asthma better than eating dirt? *Am J Respir Cell Mol Biol*. **28,** 645–647.

Silverman, N. and Fitzgerald, K. (2004). DUBbing down innate immunity. *Nat. Immunol*. **5,** 1010–1012.

Silverman, G.J. (1997). B-cell superantigens. *Immunol. Today* **18,** 379–386.

Singer, M. and Sansonetti, P.J. (2004). IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. *J. Immunol*. **173,** 4197–4206.

Smith, C.M., Wilson, N.S., Waithman, J., Villadangos, J.A., Carbone, F.R., Heath, W.R. and Belz, G.T. (2004). Cognate $CD4^+$ T cell licensing of dendritic cells in $CD8^+$ T cell immunity. *Nat. Immunol*. **5,** 1143–1148.

Soman, A., Honeybourne, D., Andrews, J., Jevons, G. and Wise, R. (1999). Concentrations of moxifloxacin in serum and pulmonary compartments following a single 400 mg oral dose in patients undergoing fiber-optic bronchoscopy. *J. Antimicrob. Chemother*. **44,** 835–838.

Soong, G., Reddy, B., Sokol, S., Adamo, R. and Prince, A. (2004). TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J. Clin. Invest*. **113,** 1482–1489.

Speert, D.P. and Goldberg, J.B. (2004). Burkholderia cepacia complex and cystic fibrosis In search of the smoking gun. *Am. J. Respir. Crit. Care. Med*. **170,** 6–7.

Stevenson, N.J., Haan, S., McClurg, A.E., Mc Grattan, M.J., Armstrong, M.A., Heinrich, P.C. and Johnston, J.A. (2004). The chemoattractants, IL-8 and Formyl-Methionyl-Leucyl- Phenylalanine, regulate granulocyte colony-stimulating factor signaling by inducing suppressor of cytokine signaling-1 expression. *J. Immunol*. **173,** 3243–3249.

Stock, P., Akbari, O., Berry, G., Freeman, G.J., DeKruyff, R.H. and Umetsu, D.T. (2004). Induction of T-helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat. Immunol*. **5,** 1149–1156.

Strieter, R.M., Belperio, J.A. and Keane, M.P. (2003). Host innate defenses in the lung: the role of cytokines. *Curr. Opin. Infect. Dis*. **6,** 193–198.

Struck, A., Forster, J., Ihorst, G., Werchau, H., König, W. and König B. (2004). Respiratory syncytial virus: G-gene genotype and disease severity. *Ped. Inf. Dis. J*. in press.

Sundrud, M.S., Torres, V.J., Unutmaz, D. and Cover, T.L. (2004). Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc. Nat. Acad. Sci. USA* **101,** 7727–7732.

Svanborg, C., Godaly, G. and Hedlund, M. (1999). Cytokine responses during mucosal infections: role in disease pathogenesis and host defense. *Curr. Opin. Microbiol*. **2,** 99–105.

Taggart, C., Coakley, R.J., Greally, P., Canny, G., O'Neill, S.J. and McElvaney, N.G. (2000). Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-alpha and interleukin-8. *Am. J. Physiol. Lung. Cell. Mol. Physiol*. **278,** L33–41.

Takeuchi, O., Hoshino, K. and Akira, S. (2000). Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus infection*. *J. Immunol*. **165,** 5392–5396.

Tiemersma, E.W., Bronzwaer, St., Lyytikäinen, O., Degener, J.E., Schrijnemakers, P., Bruinsma, N., Monen, J., Witte, W. and Grundmann, H. (2004). Methicillin-resistant Staphylococcus aureus in Europe, 1999–2002. *Emerg. Inf. Dis*. **10,** 1627–1634.

Tomita, T. and Kamio, Y. (1997). Molecular biology of the pore-forming cytolysins from Staphylococcus aureus, alpha- and gamma-hemolysins, and leukocidin. *Biosci. Biotechnol. Biochem*. **61,** 565–572.

TranVan Nhieu, G., Clair, C., Grompone, G. and Sansonetti, P. (2004). Calcium signaling during cell interactions with bacterial pathogens. *Biol. Cell*. **96,** 93–101.

Trinchieri, G. (1998). Immunobiology of interleukin-12. *Immunol. Res*. **17,** 269–278.

Trotha, R., Reichl, U., Thies, F.L., Sperling, D., König, W. and König, B. (2002). Adaption of a fragment analysis technique to an automated high-throughput multicapillary electrophoresis device for the precise qualitative and quantitative characterization of microbial communities. *Electrophoresis* **23,** 1070–1079.

Tsai, W.C, Strieter, R.M., Mehrad, B., Newstead, M.W., Zeng, X. and Standiford, T.J. (2000). CXC chemokine receptor CXCR2 is essential for protective innate host response in murine Pseudomonas aeruginosa pneumonia. *Infect. Immun*. **68,** 4289–4296.

Tvinnereim, A.R., Hamilton, S.E. and Harty, J.T. (2004). Neutrophil involvement in cross-priming $CD8^+$ T cell responses to bacterial antigens. *J. Immunol*. **173,** 1994–2002.

Uehara, A., Sugawara, Y., Sasano, T., Takada, H. and Sugawara, S. (2004). Pro-inflammatory cytokines induce proteinase 3 as membrane-bound and secretory forms in human oral epithelial cells and antibodies to proteinase 3 activate the cells through protease-activated receptor-2. *J. Immunol*. **173,** 4179–4189.

Van Vlem, B., Vanholder, R., De Paepe, P., Vogelaers, D. and Ringoir, S. (1996). Immunomodulating effects of antibiotics: literature review. *Infection* **24,** 275–291.

Vandenesch, F., Naimi, T., Enright, M.C., Lina, G., Nimmo, G.R., Heffernan, H., Liassine, N., Bes, M., Greenland, T., Reverdy, M.E. and Etienne, J. (2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg. Infect. Dis*. **9,** 978–984.

Veldkamp, K.E., Heezius, H.C.J.M., Verhoef, J., van Strijp, J.A.G. and van Kessel, K.P.M. (2000). Modulation of neutrophil chemokine receptors by Staphylococcus aureus supernate. *Infect. Immun*. **68,** 5908–5913.

Vergnolle, N., Wallace, J.L., Bunnett, N.W. and Hollenberg, M.D. (2001). Protease-activated receptors in inflammation, neuronal signaling, and pain. *Trends Pharmakol. Sci*. **22,** 146–152.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Memet, S., Huerre, M.R., Coyle, A.J., Distefano, P.S., Sansonetti, P.J., Labigne, A., Bertin, J., Philpott, D.J. and Ferrero, R.L. (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat. Immunol*. **5,** 1166–1174.

Visintin, A., Mazzoni, A., Spitzer, J.H., Wyllie, D.H., Dower, S.K. and Segal, D.M. (2001). Regulation of Toll-like receptors in human monocytes and dentritic cells. *J. Immunol*. **166,** 249–255.

Vogelmann, R., Amieva, M.R., Falkow, S. and Nelson, W.J. (2004). Breaking into the epithelial apical-junctional complex—news from pathogen hackers. *Curr. Opin. Cell. Biol*. **16,** 86–93.

Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Vuong, C., Kobayashi, S.D., Porcella, S.F., Otto, M., Musser, J.M. and DeLeo, F.R. (2004). Engagement of the pathogen survival response used by Group A Streptococcus to avert destruction by innate host defense. *J. Immunol*. **173,** 1194–1201.

Waldvogel, F. A. and Bisno, A. L. (2000). *Infections Associated with Indwelling Medical Devices* (3 ed.). ASM Press, Washington, DC.

Wang, J., Brooks, E.G., Bamford, K.B., Denning, T.L., Pappo, J. and Ernst, P.B. (2001). Negative selection of T cells by Helicobacter pylori as a model for bacterial strain selection by immune evasion. *J. Immunol*. **167,** 926–934.

Wat, D. and Doull, I. (2003). Respiratory virus infections in cystic fibrosis. *Pediatr. Respir. Rev*. **4,** 172–177.

Weinrauch, Y. and Zychlinsky, A. (1999). The induction of apoptosis by bacterial pathogens. *Annu. Rev. Microbiol*. **53,** 155–187.

Wiegers, W. Just, I., Muller, H., Hellwig, A., Traub, P. and Aktories, K. (1991). Alteration of the cytoskeleton of mammalian cells cultured *in vitro* by Clostridium botulinum C2 toxin and C3 ADP-ribosyltransferase. *Eur. J. Cell. Biol*. **54,** 237–245.

Wolf, B.B. and Green, D.R. (1999). Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation. *J. Biol. Chem*. **274,** 30651–30666.

Wong, B. and Choi, Y. (1997). Pathways leading to cell death in T cells. *Curr. Opin. Immunol*. **9,** 358–364.

Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K. and Akira, S. (2002). Essential role for TIRAP in activation of the signaling cascade shared by TLR2 and TLR4. *Nature* **420,** 324–329.

Yao, T., Mecsas, J., Healy, J.I., Falkow, S. and Chien, Y. (1999). Suppression of T and B lymphocyte activation by a Yersinia pseudotuberculosis virulence factor, yopH. *J. Exp. Med*. **190,** 1343–1350.

Ye, G., Barrera, C., Fan, X., Gourley, W.K., Crowe, S.E., Ernst, P.B. and Reyes, V. E. (1997). Expression of B7-1 and B7-2 co-stimulatory molecules by human gastric epithelial cells: potential role in CD4+ T cell activation during Helicobacter pylori infection. *J. Clin. Invest*. **99,** 1628–1636.

Yu, H. and Head, N. E. (2002). Persistent infections and immunity in cystic fibrosis. *Front. Biosci*. **7,** 442–457.

Yu, H., Hanes, M., Chrisp, C.E., Boucher, J.C. and Deretic, V. (1998). Microbial pathogenesis in cystic fibrosis: pulmonary clearance of mucoid Pseudomonas aeruginosa and inflammation in a mouse model of repeated respiratory challenge. *Infect. Immun*. **66,** 280–288.

Zhang, L., Peeples, M.E., Boucher, R.C., Collins P.L. and Pickles R.J. (2002). Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J. Virol*. **76,** 5654–5666.

Zhu, J., Min B., Hu-Li, J., Watson, C.J., Grinberg, A., Wang, Q., Killeen, N., Urban, J.F. Jr., Guo, L. and Paul, W.E. (2004) Conditional deletion of Gata3 shows its essential function in Th1-Th2 responses. *Nat. Immunol*. **5,** 1157–1165.

Zychlinsky, A. and Sansonetti, P. J. (1997). Apoptosis as a pro-inflammatory event: what can we learn from bacteria-induced cell death? *Trends Microbiol*. **5,** 201–204.

CHAPTER

# 55

# Clostridial toxins in the pathogenesis of gas gangrene

*Amy E. Bryant and Dennis L. Stevens*

## INTRODUCTION

The genus *Clostridium* encompasses over 60 species of Gram-positive anaerobic spore-forming rods that cause a variety of infections in humans and animals. Strains of clostridia, such as *C. perfringens, C. histolyticum, C. septicum*, *C. novyi*, and *C. sordellii,* cause aggressive necrotizing or histotoxic infections of the soft tissues, attributable in part to the elaboration of bacterial proteases, phospholipases, and cytotoxins (Table 55.1). Historically, the major lethal toxins of the clostridia were assigned Greek letters, with the letter "α" always used to designate the most potent or most significant lethal factor (Table 55.1). A marvelous review of these data can be found in the monograph by Smith (Smith, 1975b). Over the last 50 years, modern science has provided a greater understanding of mecha nisms of action of some of these factors.

The term "histotoxic clostridial infection" historically referred to gas gangrene and malignant edema in humans and blackleg in cattle. More recently, novel histotoxic infections have been described, such as necrotic enteritis, neutropenic enterocolitis, and spontaneous gas gangrene—all of which occur exclusively in humans—and abomasal ulceration in cattle (Table 55.1). In each case, the infection is rapidly progressive, associated with gas in tissue, and manifests impressive tissue destruction, shock, and frequently death. This review chapter will discuss briefly the clinical characteristics of some of these infections and will describe the role of extracellular toxins in the manifestations of disease.

## THE MAJOR HISTOTOXIC CLOSTRIDIAL INFECTIONS

### Traumatic gas gangrene

Gas gangrene, or clostridial myonecrosis, is one of the most fulminant Gram-positive infections of humans. A well-known complication of battlefield injuries, clostridial gas gangrene can develop rapidly (within 6–8 hrs) following any trauma that is sufficient to compromise the blood supply (crush injury, gunshot or knife wound, open fractures). *Clostridium perfringens* is isolated in over 80% of such infections, the remaining cases being caused by *C. septicum*, *C. novyi* type A, *C. histolyticum*, and *C. bifermentans*. Gas gangrene is characterized by excruciating pain at the infection site (MacLennan, 1962), the onset of which is "sometimes so sudden as to suggest a vascular catastrophe" (MacLennan, 1962). Destruction of healthy tissue can occur at a rate of several inches per hour despite appropriate antibiotic therapy (McNee and Dunn, 1917; MacLennan, 1962), and radical amputation remains the single best lifesaving treatment. Shock and organ failure frequently accompany gas gangrene and when patients become bacteremic, the mortality exceeds 50%.

The mechanisms responsible for the early onset of severe pain and the rapid regional destruction of tissues in this infection have not been completely elucidated; however, toxin-mediated microvascular thrombosis contributes to reduced tissue perfusion,

**TABLE 55.1** Major virulence factors of the histotoxic clostridia

| Clinical Infection | Organism | Virulence Factor | Mechanism of Action |
|---|---|---|---|
| Traumatic gas gangrene | *Clostridium perfringens* Type A | alpha toxin | phospholipase C |
| | | theta toxin | cholesterol-dependent cytolysin |
| | | kappa toxin | collagenase |
| | | mu toxin | hyaluronidase |
| | | nu antigen | deoxyribonuclease |
| Traumatic gas gangrene | *Clostridium histolyticum* | alpha toxin | non-hemolytic, cytotoxic, lethal, antigenically related to alpha toxin of *C. septicum* |
| | | beta toxin | collagenase |
| | | gamma toxin | thiol-activated protease |
| | | delta toxin | elastase |
| | | epsilon toxin | thiol-activated, cholesterol-binding cytolysin |
| Traumatic gas gangrene | *Clostridium novyi* | alpha toxin | large clostridial cytotoxin with glycosyltransferase activity; modifies small GTPases controlling actin cytokeleton; dermonecrotic, causes gelatinous edema |
| | | gamma toxin | phospholipase C |
| | | delta toxin | thiol-activated, cholesterol-dependent cytolysin |
| Traumatic or spontaneous gas gangrene; Neutropenic enterocolitis in humans | *Clostridium septicum* | alpha toxin | cytotoxic, lethal, hemolytic, antigenically related to alpha toxin of *C. histolyticum* |
| | | beta toxin | deoxyribonuclease |
| | | gamma toxin | hyaluronidase |
| | | delta toxin | cholesterol-dependent cytolysin |
| Gas gangrene and malignant edema | *Clostridium sordellii* | alpha toxin | phospholipase C |
| | | beta toxin | "edema producing factor" |
| | | gamma toxin | protease |
| | | ? | phospholipase A |
| | | ? | lysolecithinase |
| | | ? | thiol-activated cytolysin |
| | | ? Lethal Toxin (LT) and Hemorrhagic Toxin (HT) | large clostridial cytotoxins with glycosyltransferase activity; modifies small GTPases controlling actin cytokeleton |
| Enteritis necroticans | *Clostridium perfringens* Type C | beta toxin | cytolytic for intestinal villi |
| Abomasal ulceration in cattle | *Clostridium perfringens* Type A | see above | |
| Blackleg in cattle | *Clostridium chauvoei* | alpha toxin | lethal, necrotizing |
| | | beta toxin | deoxyribonuclease |
| | | gamma toxin | hyaluronidase |
| | | delta toxin | thiol-activated cytolysin |

hypoxia, and subsequent regional tissue necrosis. Clinical observations support this concept. First, the speed with which skin, subcutaneous tissue, fascia, and muscle are destroyed in gas gangrene is similar to the rate of tissue death following acute arterial thrombosis. Second, intense pain is a prominent feature in clinical conditions that involve occlusion of the arterial blood supply, such as myocardial infarction. Third, tissues that are being rapidly destroyed in the progression of gas gangrene do not bleed. This classic observation has become dictum for surgeons who routinely remove necrotic tissue until bleeding is encountered. Indeed, tissues from experimental animals in the early stages of gas gangrene demonstrate numerous occlusive thrombi throughout adjacent musculature. Taken together, these observations suggest that severe pain and rapid tissue destruction associated with clostridial gas gangrene result from vascular occlusion that begins as a local ischemic process and expands regionally until an entire limb is destroyed.

## Spontaneous, non-traumatic gas gangrene due to *Clostridium septicum*

Spontaneous gas gangrene is characterized by the abrupt onset of excruciating pain and a heavy sensation or numbness of the affected limb (MacLennan, 1962; Alpern and Dowell, Jr., 1969; Smith, 1975b; Stevens *et al*., 1990; Johnson *et al*., 1994). In this instance, *C. septicum* is the principal etiologic agent. Extremely rapid progression of gangrene follows, with demonstrable gas in the tissues, marked swelling, and the

appearance of hemorrhagic bullae in the overlying skin. The skin itself often has a purple hue, likely reflecting vascular compromise resulting from bacterial toxins diffusing into surrounding tissues (Stevens *et al*., 1990). Histopathology of the affected soft tissues demonstrates cell lysis, gas formation, and a notable absence of acute inflammatory cells (Stevens *et al*., 1990). The mortality of spontaneous gangrene ranges from 67–100%, with the majority of deaths occurring within 24 hours of onset. Predisposing host factors include colonic carcinoma, diverticulitis, and gastrointestinal surgery—pathologies that permit bacterial access from the intestine to the bloodstream from which the aerotolerant *C. septicum* can seed and proliferate in normal tissues (Smith, 1975b).

*C. septicum* produces four main toxins (Table 55.1): alpha toxin (α, lethal, hemolytic, necrotizing activity); beta toxin (β, DNase); gamma toxin (γ, hyaluronidase); and delta toxin (δ, septicolysin, an oxygen labile hemolysin) (Smith, 1975b). The *C. septicum* alpha toxin does not possess phospholipase activity and is thus distinct from the alpha toxin of *C. perfringens*. Active immunization against alpha toxin significantly protects against challenge with viable *C. septicum* (Ballard *et al*., 1992). The alpha toxin is secreted as an inactive prototoxin that is cleaved near the C-terminus by eukaryotic proteases, such as trypsin or membrane-bound furin, to form the active toxin (Gordon *et al*., 1997). The toxin then oligomerizes on the membrane and inserts, forming a membrane pore and resulting in colloid-osmotic lysis (Sellman *et al*., 1997). It is unclear at this point in time just how these virulence factors cause tissue necrosis.

## Necrotizing enteritis

Defects in the immune system, such as leukemia, neutropenia (cyclic, congenital, or acquired), lymphoproliferative disorders, and more recently, AIDS (Alpern and Dowell, Jr., 1969; Stevens *et al*., 1990; Johnson *et al*., 1994) or those induced by cancer chemotherapy and radiation therapy, also predispose to *C. septicum* infection (Farnell, 1987; Bartlett, 1990; Gorbach, 1992). Neutropenia in particular is associated with necrotizing enterocolitis (Farnell, 1987)—a fulminant form of enteritis characterized by copious watery diarrhea, abdominal pain, and distention, and systemic signs of toxicity such as tachycardia, fever, and delirium. Rupture of the bowel with peritonitis and bacteremia results in death in 100% of cases. In most cases, immunofluorescence studies demonstrate invasion of the bowel wall by *C. septicum*.

Other forms of necrotizing enteritis have occurred endemically in New Guinea (e.g., pigbel, Lawrence and Walker, 1976), in epidemic proportion in Germany following World War II (Guerrant, 1990), and sporadically in Africa, Southeast Asia, and the United States (Bartlett, 1990; Gorbach, 1992). All cases are associated with the ingestion of meats contaminated with *C. perfringens* type C. Clinical courses vary between abdominal pain, fever, and diarrhea, which resolves spontaneously, to bloody diarrhea, ruptured bowel, and death. Beta toxin from *C perfringens* type C, which paralyzes the intestinal villi and causes necrosis of the bowel wall, is the principal cause of these infections. Predisposing factors include malnutrition, specifically in those with diets low in protein and rich in trypsin inhibitors, such as sweet potato or soy bean (Lawrence and Walker, 1976; Guerrant, 1990). In addition, *Ascaris lumbricoides* is commonly found in such patients, and it, too, secretes a trypsin inhibitor. These protease inhibitors protect beta toxin from intraluminal proteolysis. Immunization of children in New Guinea with a beta toxoid vaccine has dramatically reduced the incidence of this disease (Lawrence *et al*., 1979).

## Lethal, necrotizing clostridial infections in injecting drug users

Recently, outbreaks of severe soft tissue infection have been described among extravascularly injecting drug (heroin) users in Scotland, Ireland, England, and the United States (Centers for Disease Control and Prevention, 2000; McGuigan *et al*., 2002; Jones *et al*., 2002; Kimura *et al*., 2004). Clinical findings included severe injection site necrosis with massive edema and a rapidly fatal systemic illness characterized by hypotension, pulmonary edema, pericardial and pleural effusions, and leukocytosis. Of the 23 definite cases described in the Scottish outbreak, 15 had necrotizing fasciitis, and 20 died (McGuigan *et al*., 2002). Among these, *C. novyi* Type A and *C. perfringens* were the principal isolates (Centers for Disease Control and Prevention, 2000). More recently, similar cases in Europe have been attributed to *C. histolyticum* (Brazier *et al*., 2004). Of the nine cases described in California (Kimura *et al*., 2004), all developed necrotizing fasciitis, marked leukocytosis and hypotension, and four died. Cultures from six of the patients yielded *C. sordellii*. While the mechanisms resulting in necrotizing fasciitis in these infections remain unknown, soft tissue and pulmonary edema, hypotension, and hemoconcentration are likely due to capillary endothelial cell disruption by related members of the large clostridial cytotoxin family, including *C. novyi* alpha toxin and *C. sordellii* hemorrhagic and lethal toxins (Bette *et al*., 1991). These toxins possess glucosyltransferase activity and specifically modify small GTPases (e.g., Rho, Rac, Cdc42),

controlling actin polymerization (Schirmer and Aktories, 2004). In contrast, edema associated with cases of *C. histolyticum* infection may be related to beta toxin (i.e., collagenase)–induced production of bradykinin (Legat *et al*., 1994).

## THE ROLE OF EXOTOXINS IN C. *PERFRINGENS* GAS GANGRENE

### The major C. *perfringens* exotoxins

Of the myriad of extracellular toxins produced by *C. perfringens* (Table 55.1), only alpha and theta toxins have been implicated in pathogenesis. Alpha toxin is a lethal, hemolytic toxin that has both phospholipase C and sphingomyelinase activities. Two recent independent studies have demonstrated that alpha toxin is an essential toxin in the disease process. First, an alpha toxin gene (*plc*) deficient mutant was shown to be avirulent in a mouse myonecrosis model (Awad *et al*., 1995). Complementation of the chromosomal mutation with a recombinant plasmid carrying a wild-type *plc* gene restored the ability to cause disease. Second, vaccination with the C-terminal alpha toxin domain (amino acids 247 to 370) has been shown to protect mice from experimental *C. perfringens* infection (Williamson and Titball, 1993) in part, by improving tissue perfusion and restoring the tissue inflammatory response (Stevens *et al*., 2004).

Theta toxin (also known as perfringolysin O, PFO) is a member of the thiol-activated, now termed cholesterol-dependent, cytolysin family (CDCs) that includes streptolysin O, pneumolysin, listeriolysin, septicolysin, and others (Alouf and Geoffroy, 1991). These pore-forming toxins contain a conserved ECTGLAWEWWR amino acid motif. Despite its hemolytic activity, theta toxin, by itself, is not essential in causing mortality since isogenic mutant strains lacking an intact theta toxin structural gene (*pfoA*) are still lethal in the mouse myonecrosis model (Awad *et al*., 1995). However, these same genetic studies, as well as passive immunization studies in mice (Bryant *et al*., 1993), suggest that theta toxin does contribute to pathogenesis by enhancing the morbidity associated with gas gangrene, likely via its effects on cells of the vascular and immune systems (Bryant *et al*., 1993; Bryant and Stevens, 1996; Stevens *et al*., 1997).

### Stages in the pathogenesis of C. *perfringens* gas gangrene

The development of gas gangrene occurs in five distinct stages, and alpha and theta toxins feature prominently in disease progression.

#### *Stage 1: Introduction of* C. perfringens *into devitalized tissue*

Vegetative clostridia or their spores often contaminate traumatic wounds; however, proliferation of *C. perfringens* in tissue requires trauma sufficient to interrupt the blood supply to large muscle groups. Loss of perfusion rapidly results in a drop in oxidation reduction potential from +170 to +50 mV, which, at neutral pH, is sufficient for growth of most anaerobic bacteria including the clostridia. Hypoxic muscle shifts to anaerobic metabolism, producing a lactic acid–driven increase in hydrogen ion concentration that allows clostridia to proliferate at oxidation reduction potentials higher than +50 mV (Smith, 1975b). In addition, hypoxia induces release of endogenous lysosomal enzymes that convert muscle protein into peptides that are vital to clostridial growth and toxin production (Smith, 1975a). Interestingly, intramuscular injection of recombinant alpha toxin in experimental animals results in a major drop in local pH as measured by nuclear magnetic resonance imaging (D.L. Stevens, unpublished observations).

#### *Stage 2: Proliferation of organisms*

As organisms begin to proliferate locally, end products of bacterial metabolism further reduce tissue pH, and bacterial proteases continue tissue degradation, thereby optimizing conditions for continued growth and toxin production. Production of *C. perfringens* alpha toxin increases in the presence of (i) peptides containing glycine and branched chain amino acids such as valine, or tyrosine-containing peptides of low molecular weight; (ii) a carbohydrate source of starch, dextran, or fructose, but not glucose; (iii) pH below 7.0; (iv) total electrolyte concentration of 0.1–0.15 M with a ratio of sodium to potassium of 2:1.

#### *Stage 3: Attenuation of the tissue inflammatory response*

Histologically, gas gangrene is characterized by the virtual absence of a tissue inflammatory response and is thus distinctly different from infections caused by bacteria such as *Staphylococcus aureus*, *Hemophilus influenzae*, or *Streptococcus pneumoniae* in which an abundant pyogenic tissue inflammatory response is the norm. Studies performed during the first half of the twentieth century suggested that this characteristic finding was bacterial toxin–mediated (McNee and Dunn, 1917). A 1917 report in the *British Medical Journal* described the histopathology of serial sections of single muscle bundles from freshly amputated limbs of soldiers with gas gangrene (McNee and Dunn, 1917). At the advancing edge of the infection, few organisms were present and muscle fibers appeared ischemic and necrotic,

being degraded by a toxic fluid that spread between the fibers in advance of the organisms themselves. Leucocytes were "generally conspicuous by their absence" from involved tissues but were seen in the interfascial planes (McNee and Dunn, 1917). In this region, leukocytes exhibited altered morphology and karyolysis (Robb-Smith, 1945), suggesting that the clostridial exotoxins were directly cytotoxic.

This characteristic lack of a tissue inflammatory response in *C. perfringens* gas gangrene is, at first, surprising since the organism is known to activate the complement cascade with the generation of serum-derived chemotactic factors and opsonins via the alternative complement pathway (Stevens *et al*., 1987). In addition, alpha toxin is known to stimulate production of the potent chemokine interleukin-8 (IL-8) (Bryant and Stevens, 1996) and to up-regulate endothelial cell adherence molecules necessary for diapedesis (Bryant and Stevens, 1996; Bunting *et al*., 1997). Thus, a normal inflammatory response to *C. perfringens* infection should result. Indeed, this initial immune response likely arrests bacterial proliferation in most patients since progression to full-blown clinical gas gangrene develops in only 0.03–5.2% of traumatic open wounds despite the fact that 4–39% of these wounds are contaminated with histotoxic clostridial species (Altemeier and Fullen, 1971).

However, in those patients who develop clinically recognized clostridial gas gangrene (Stevens, 1995), and in experimental wild-type *C. perfringens* infection (Bryant *et al*., 1993), the lack of a tissue inflammatory response and concomitant vascular leukostasis are hallmark features. Alpha and theta toxins each uniquely contribute to these pathologies. Experimental histopathology studies demonstrated that the robust tissue inflammatory response to killed, washed *C. perfringens* could be completely inhibited by either a crude clostridial toxin preparation (Robb-Smith, 1945), or recombinant clostridial alpha (Bryant *et al*., 1995) or theta toxins (Bryant *et al*., 1993). Instead, leukocytes accumulated within small vessels near the demarcation between healthy and necrotic tissues (Bryant *et al*., 1993). Similarly, roles for both theta and alpha toxins in the anti-inflammatory response has been demonstrated in studies utilizing isogenic mutants strains of *C. perfringens* lacking these toxins (Awad *et al*., 1995; Stevens *et al*., 1997). More recently, immunization against the C-domain of alpha toxin, which was 100% protective against live challenge, restored the local inflammatory response (Stevens *et al*., 2004) (Figure 55.1). Since production of both alpha and theta toxins occurs at the site of the experimental infection by 4 hrs (Noyes *et al*., 1964), the earliest events of the initial encounter of the host immune system with the pathogenic organism determine the outcome of infection.

The mechanism of attenuation of the host's inflammatory response can be inferred from *in vitro* studies demonstrating direct and unique concentration-dependent effects of alpha and theta toxins on leukocyte structure and function. Specifically, low concentrations of theta toxin prime PMNL for increased respiratory burst activity and superoxide anion generation (Stevens *et al*., 1987; Bryant *et al*., 1993) and enhance ingestion of complement-opsonized particles due to theta toxin–induced functional up-regulation of complement receptor 3 (CR3, CD11b/CD18) on neutrophils (Bryant

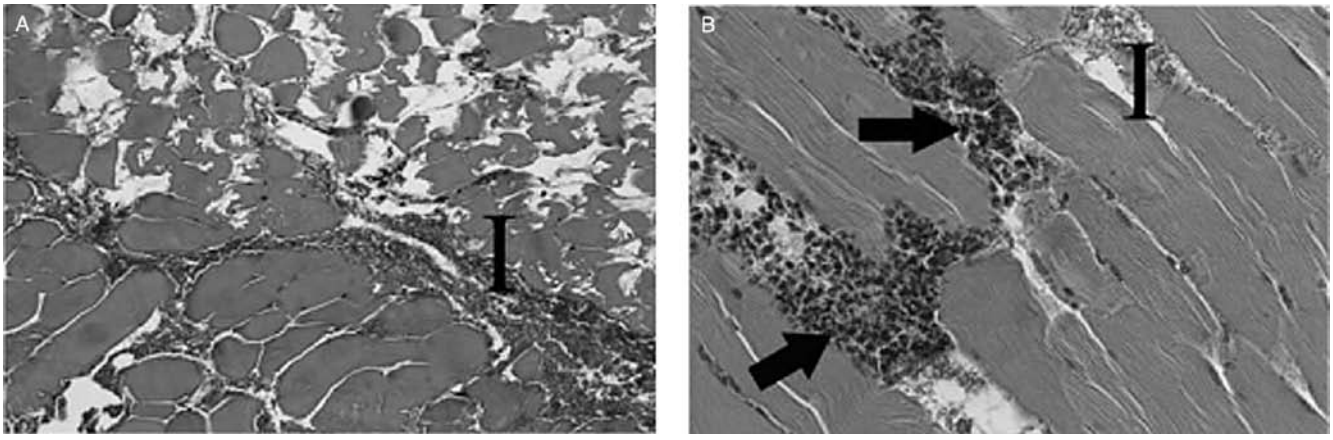



**FIGURE 55.1** Immunization with the C-domain of *C. perfringens* PLC restores the tissue inflammatory response. Mice were immunized against adjuvant alone (sham) or adjuvant plus the C-domain of *C. perfringens* PLC and then infected intramuscularly with $3.75\times10^8$ washed, log phase *C. perfringens* ATCC 13124 in the right thigh. At 1 hr post infection, tissue from the infected site was harvested and processed for routine hematoxylin/eosin staining. Sham-immunized animals (A) had extensive tissue necrosis and infiltrating PMNL were absent. In contrast, in the C-domain immunized animals (B), muscle had a relatively good appearance and PMNL influx was clearly visible. I = bacterial inoculum. Solid arrows indicate infiltrating PMNL.

*et al*., 1993). At higher concentrations, theta toxin significantly impairs these functions, decreases PMNL viability (Stevens *et al*., 1987), induces a hyperadhesive state (Stevens *et al*., 1987; Bryant *et al*., 1993), and causes desensitization of chemotactic factor receptors (Bryant and Stevens, 1996). These latter two effects specifically abrogate neutrophil directed migration.

The effects of alpha toxin on phagocyte function are no less remarkable. Alpha toxin primes resting PMNL respiratory burst in response to opsonized zymosan with resultant maximal generation of oxygen radical production (Stevens *et al*., 1987; Bryant *et al*., 1991). Morphologically, PMNL treated with alpha toxin assume a cytokineplast form with multiple daughter cells connected by a cytoplasmic bridge (Stevens *et al*., 1989; Bryant *et al*., 1991). Premature priming of cells derails anaerobic glycolysis in favor of hexose monophosphate shunt activity, subverting generation of ATP necessary for the cytoskeletal rearrangements involved in chemotaxis and ingestion.

The net effect is an inhibition of the tissue inflammatory response and an increase in the adherence of highly activated PMNL to vascular endothelium—a scenario that not only leaves the infection unchecked, but also promotes vascular injury.

### *Stage 4: Progression of local and regional tissue destruction*

*Platelet-mediated microvascular thrombosis*

Platelet aggregation is a well-known biological effect of *C. perfringens* alpha toxin, having been described since the mid-1970s (Sugahara *et al*., 1976). Topical application of alpha toxin to the mesentery of rats revealed platelet thrombi in adjacent vessels that occluded mesenteric blood flow, leading the investigators to conclude that thrombosis was likely an early step in the pathogenesis of necrosis due to alpha toxin (Ohsaka *et al*., 1978). Similarly, we have recently demonstrated that intramuscular injection of *C. perfringens* alpha toxin induced a rapid (less than 2 min) and irreversible decline in skeletal muscle perfusion that occurred concomitantly with the formation of intravascular aggregates of activated platelets, leukocytes, and fibrin (Bryant *et al*., 2000b) (Figure 55.2). These aggregates were freely moving until achieving sufficient size to completely and irreversibly occlude capillaries, venules, and arterioles (Figure 55.3). Only at the later stages did these heterotypic aggregates appear fixed to the endothelium (Bryant *et al*., 2000a; Bryant *et al*., 2000b).

This sequence of events was strikingly different from that which occurs following traumatic vessel injury in which tethering of platelets to the injured site is mediated by platelet gpIbα binding to von Willebrand factor (Ruggeri, 1997). This adhesion then stimulates "outside-in" signaling events that result in activation of the platelet fibrinogen receptor gpIIbIIIa (Lipfert *et al*., 1992; Law *et al*., 1996) and in redistribution of platelet membrane phospholipids. These events promote the adherence of additional platelets and interactions with elements of the clotting cascade to generate a highly localized clot.

However, the appearance of non-adherent, freely mobile and growing platelet/leukocyte aggregates suggested that alpha toxin induces cellular aggregation

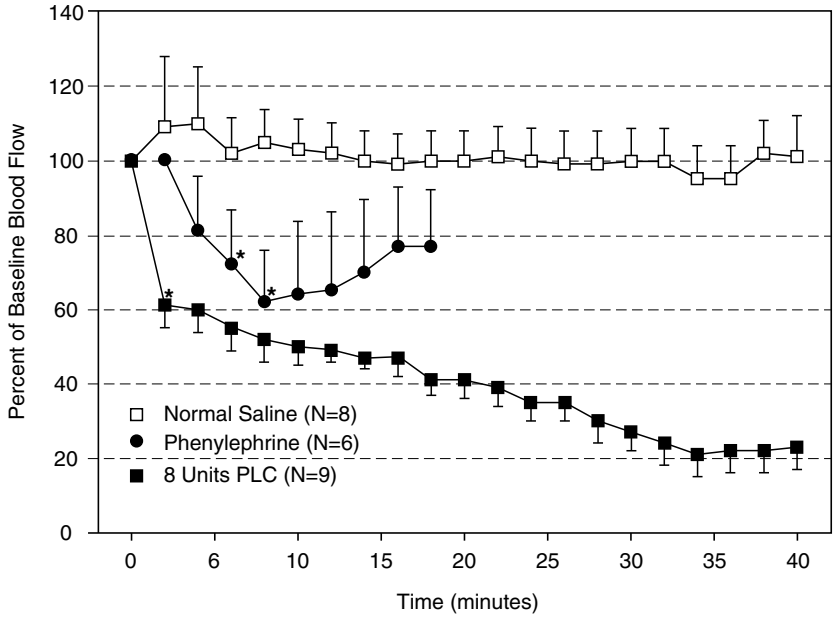



**FIGURE 55.2** Intramuscular injection of clostridial toxins irreversibly reduces muscle perfusion. Rat abdominal muscles were injected with 0.1 mL of either normal saline, a clostridial toxin preparation diluted to contain 8 units of PLC activity, or the vasoconstrictor phenylephrine. Blood flow was measured by a laser Doppler blood perfusion monitor and was expressed as the percent of baseline perfusion ± standard error. Phenylephrine transiently reduced perfusion. In contrast, toxin-induced perfusion deficits were progressive and irreversible.

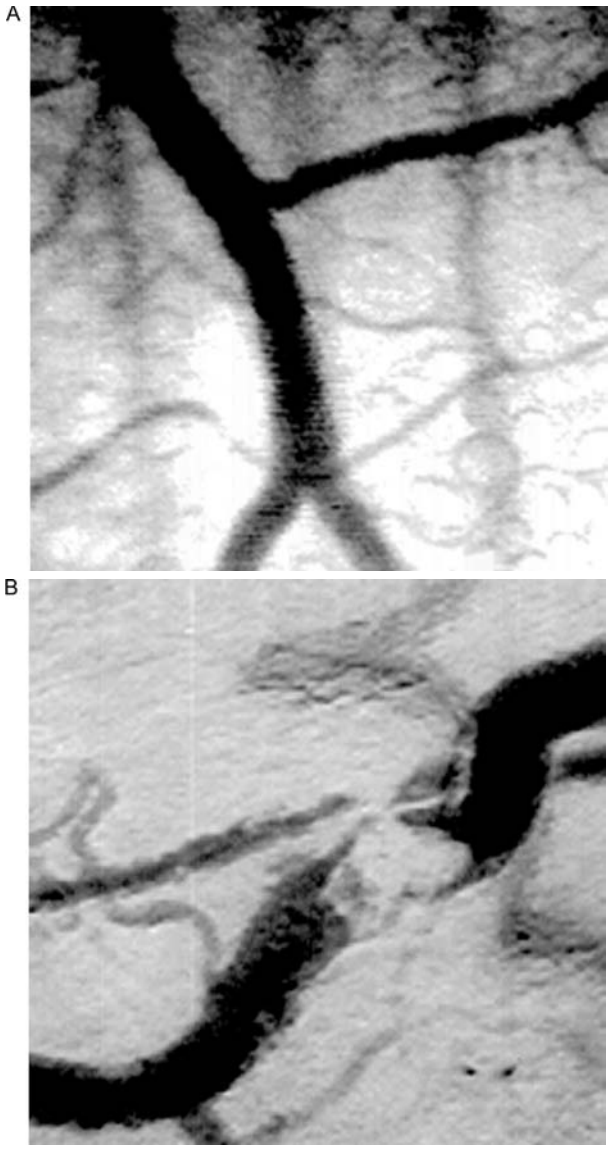



**FIGURE 55.3** Toxin-induced intravascular aggregates block blood flow in venules and arterioles. Still image of intravascular aggregates in vessels taken from representative videotapes following intramuscular injection of saline or a clostridial toxin preparation containing 8 U PLC. Venule under baseline conditions (i.e., before toxin injection); magnification = 210× (A). Venule and arteriole at 11 min after toxin injection (B). Both the large venule and the adjacent smaller arteriole are occluded by large stationary aggregates; magnification = 267×.

in a completely different manner. For circulating platelets to co-aggregate and to bind other cell types, a conformational change in platelet gpIIbIIIa is necessary to permit fibrinogen binding (Blockmans *et al*., 1995). Indeed, flow cytometric analyses of alpha toxin–treated whole blood demonstrated that the formation of both platelet/platelet and platelet/leukocyte aggregates was mediated by the activation of platelet gpIIbIIIa (Bryant *et al*., 2000a). Alpha toxin–induced activation of gpIIbIIIa was highly calcium dependent since pharmacologic inhibitors of intracellular calcium release and of store-operated plasma membrane calcium channels blocked gpIIbIIIa activation (Bryant *et al*., 2003). Surprisingly, alpha toxin–induced activation of gpIIbIIIa was protein kinase C (PKC)–independent despite the fact that PKC was strongly activated by alpha toxin treatment (Bryant *et al*., 2003). This finding was unexpected since most agonists that directly or indirectly activate PKC also activate gpIIbIIIa. However, treatment of platelets with *C. perfringens* alpha toxin resulted in the proteolytic cleavage of soluble PKC into a calcium- and phosphospholipid-independent, but enzymatically active, low molecular weight form of the kinase (Tapley and Murray, 1984) that does not support gpIIbIIIa activation.

Thus, alpha toxin initiates an "inside-out" signaling cascade that begins with depletion of internal calcium stores, is sustained by an influx of calcium through store-sensitive channels, and culminates in functional activation in gpIIbIIIa. Further, the lack of involvement of PKC suggests that the response to alpha toxin does not follow receptor-linked, G-protein-mediated signaling with inositol trisphosphate generation. This conclusion is further supported by the fact that $PGE_1$, a known inhibitor of Gαi-mediated signaling, had no effect on alpha toxin-induced activation of gpIIbIIIa (Bryant *et al*., 2003). Instead, neutralization studies with monoclonal antibody against alpha toxin suggest that activation of gpIIbIIIa is a direct consequence of the toxin's phospholipase C and/or sphingomyelinase activities and not receptor occupancy. This model does not, however, exclude a potentiating role of other mediators (e.g., thromboxane production) in the alpha toxin–induced effects.

*Leukostasis and vascular injury*

Experimental studies (Bryant *et al*., 1993; Stevens *et al*., 1997), as well as classical clinical observations (McNee and Dunn, 1917), demonstrate not only an absence of PMNL at the site of active infection (see above), but also accumulation of PMNL within the post-capillary venules. Binding of neutrophils to endothelial cells is a multi-step process involving numerous adherence molecules on both cell types, and while many of these are required for normal margination and diapedesis, hyperadhesion of neutrophils promotes vascular injury and disrupts chemotaxis. *In vitro* studies suggest that theta toxin and alpha toxin each up-regulate early (i.e., rapidly expressed) endothelial adherence molecules, P-selectin (Bunting *et al*., 1997; Bryant *et al*., 2000b), and platelet activating factor (PAF) (Whatley *et al*., 1989; Bunting *et al*., 1997). Theta toxin-induced

PAF synthesis follows phospholipase $A_2$ ($PLA_2$) activation induced by the influx of calcium through membrane pores formed by theta toxin. $PLA_2$ hydrolysis of phosphatidylcholine releases arachidonic acid, and subsequent incorporation of acetyl-CoA into the Sn-2 position of lysophosphatidylcholine yields PAF (Whatley *et al*., 1989). Alpha toxin, via its sphingomyelinase and phospholipase C activities, produces the intracellular messengers ceramide (Ochi *et al*., 2004) and diacylglycerol, leading to activation of protein kinase C and generation of PAF (Bunting *et al*., 1997).

In contrast to the rapidly expressed endothelial cell adherence molecules, alpha and theta toxins have dichotomous effects on those adherence molecules that required *de novo* protein synthesis. Specifically, alpha toxin markedly up-regulates both E-selectin and ICAM-1 by 4 and 12 hr, respectively, whereas theta toxin stimulates only ICAM-1 expression (Bryant and Stevens, 1996). Additionally, theta toxin functio nally and quantitatively up-regulates neutrophil CD11b/CD18 (Bryant *et al*., 1993) (Figure 55.4). Coincubation of PMNL and endothelial cells in the presence of theta toxin demonstrates tight adherence of PMNL to endothelium via a CD11b/ CD18 mechanism (Bryant *et al*., 1993). Occurring *in vivo*, such adherence could attenuate blood flow and damage endothelial cells by neutrophil-dependent mechanisms such as degranulation (release of hydrolytic enzymes) and oxygen radical production (Stevens *et al*., 1997).

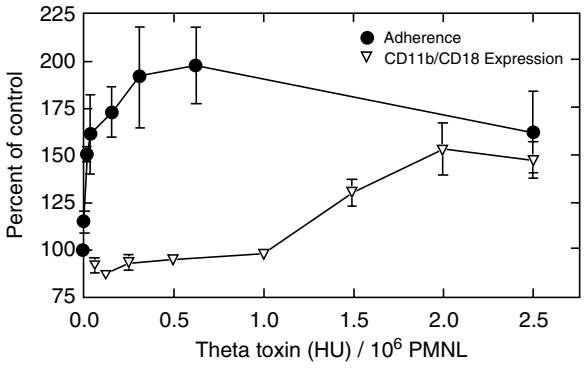



**FIGURE 55.4** Theta toxin functionally up-regulates neutrophil CD11b/CD18. Purified PMNL were exposed to increasing concentrations of recombinant theta toxin. Adherence of PMNL to gelatin-coated tissue culture wells (a CD11b-dependent event) and surface expression of CD11b were measured by Rose Bengal staining and flow cytometry, respectively. Functional up-regulation of CD11b (i.e., increased adherence) preceded its quantitative increases in surface expression induced by theta toxin.

***Stage 5: Shock and organ failure***

Cardiovascular collapse and end organ failure occur late in the course of gas gangrene. "Tachycardia with feebleness of the pulse has followed the onset of pain and characteristically, has been out of proportion to the degree of elevation of the temperature" (Altemeier and Fullen, 1971). This description offers a striking contrast to the early manifestations of septic shock caused by Gram-negative bacteria such as *Escherichia coli*, where a hyperdynamic picture is most common even in the face of low blood pressure. For example, a rapid heart rate is generally associated with high cardiac outputs and a bounding pulse. In gas gangrene, at the onset of tachycardia, the blood pressure is normal but then drops precipitously. Though these descriptions made in the 1970s are useful, the subsequent clinical literature has not offered more modern measurements of cardiovascular parameters in humans with clostridial gas gangrene. Experimental studies in animals do provide some useful insights into the dynamics of cardiovascular dysfunction induced by *C. perfringens* toxins.

In an awake rabbit model, recombinant theta toxin caused a significant increase in heart rate within 60 min of toxin infusion (Asmuth *et al*., 1995), but did not cause a significant drop in mean arterial pressure. Recombinant alpha toxin caused a significant reduction in blood pressure, but the onset of alpha toxin–induced hypotension was one hour later (i.e., at 180 min post toxin infusion) than that caused by the crude toxin (120 min post toxin). These data suggest additive or even synergistic interactions of alpha and theta toxins on cardiovascular function.

Both recombinant alpha toxin and crude toxin caused significant drops in cardiac index as early as 90 min after infusion of the respective toxins (Asmuth *et al*., 1995), suggesting that one mechanism of cardiovascular dysfunction induced by recombinant alpha toxin could be related to direct myocardial toxicity. Indeed, a direct and dose dependent reduction in myocardial contractility (dF/dt) was found in isolated atrial strips bathed with recombinant alpha toxin (Asmuth *et al*., 1995). Alpha toxin has also been shown to affect the ionotropic cardiac response in isolated embryonic chick heart preparations (Regal and Shigeman, 1980). However, alpha toxin may also contribute indirectly to shock by stimulating production of endogenous mediators, such as tumor necrosis factor (TNF) (Stevens and Bryant, 1997) and PAF (Bunting *et al*., 1997).

Theta toxin reduces systemic vascular resistance and markedly increases cardiac output (Stevens *et al*., 1988; Asmuth *et al*., 1995). This afterload reduction occurs undoubtedly through induction of endogenous

mediators that cause relaxation of blood vessel wall tension, such as PAF and prostaglandin $I_2$ ($PGI_2$) (Whatley *et al*., 1989). Reduced vascular tone develops rapidly, and in order to maintain adequate tissue perfusion, a compensatory host response is required to either increase cardiac output or rapidly expand the intravascular blood volume. In contrast, patients with Gram-negative sepsis compensate for hypotension by markedly increasing cardiac output; however, this adaptive mechanism may not be possible in *C. perfringens*-induced shock due to direct suppression of myocardial contractility by alpha toxin (Stevens *et al*., 1988). Theta toxin, like other thiol-activated cytolysins, likely contributes to septic shock through additional indirect routes, including augmented release of TNF, interleukin-1 (IL-1), and interleukin-6 (IL-6) (Houldsworth *et al*., 1994). The roles of TNF, IL-1, and IL-6, as well as the potent endogenous vasodilators bradykinin and nitric oxide, in shock associated with *C. perfringens* gas gangrene, have not been elucidated.

## CONCLUSION

In summary, in the absence of a host response sufficient to limit bacterial growth, clostridial proliferation is rapid and leads to the local accumulation of toxin. Small amounts of toxin contribute to microvascular thrombosis, reduced perfusion, and extension of the anaerobic environment, whereas higher concentrations inhibit PMNL influx and promote vascular leukostasis. At these concentrations, direct cytotoxicity is observed in emigrated leukocytes, vascular endothelium, and cells of muscle and soft tissues. As more toxin is absorbed, the series of events is propagated regionally until late in the course when the myocardium itself is directly affected, resulting in cardiovascular collapse and death.

## ACKNOWLEDGMENTS

This work was supported by grants to AEB and DLS from the Office of Research and Development, Medical Research Service, United States Department of Veterans Affairs.

## REFERENCES

Alouf, J.E. and Geoffroy, C. (1991). The Family of the Antigenically-Related, Cholesterol-Binding ("Sulphydryl-Activated") Cytolytic Toxins. In: *Sourcebook of Bacterial Protein Toxins* (eds. J.E.Alouf and J.H.Freer), pp. 147–186. Academic Press, New York.

Alpern, R.J. and Dowell, V.R., Jr. (1969). *Clostridium septicum* infections and malignancy. *JAMA* **209,** 385–388.

Altemeier, W.A. and Fullen, W.D. (1971). Prevention and treatment of gas gangrene. *JAMA* **217,** 806–813.

Asmuth, D.A., Olson, R.D., Hackett, S.P., Bryant, A.E., Tweten, R.K., Tso, J.Y., Zollman, T. and Stevens, D.L. (1995). Effects of *Clostridium perfringens* recombinant and crude phospholipase C and theta toxins on rabbit hemodynamic parameters. *J. Infect. Dis*. **172,** 1317–1323.

Awad, M.M., Bryant, A.E., Stevens, D.L. and Rood, J.I. (1995). Virulence studies on chromosomal α-toxin and θ toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of α-toxin in *Clostridium perfringens*-mediated gas gangrene. *Mol. Microbiol*. **15,** 191–202.

Ballard, J., Bryant, A., Stevens, D. and Tweten, R.K. (1992). Purification and characterization of the lethal toxin (alpha-toxin) of *Clostridium septicum*. *Infect. Immun*. **60**(3), 784–790.

Bartlett, J.G. (1990). Gas Gangrene (Other Clostridium-Associated Diseases). In: *Principles and Practice of Infectious Diseases* (eds. G.L. Mandell, R.G. Douglas and J.E. Bennett), pp. 1851–1860. Churchill Livingstone, New York.

Bette, P., Oksche, A., Mauler, F., von Eichel-Streiber, C., Popoff, M.R. and Habermann, E. (1991). A comparative biochemical, pharmacological, and immunological study of *Clostridium novyi* alpha-toxin, C. difficile toxin B and *C. sordellii* lethal toxin. *Toxicon* **29,** 877–887.

Blockmans, D., Deckmyn, H. and Vermylen, J. (1995). Platelet activation. *Blood Rev*. **9,** 143–156.

Brazier, J.S., Gal, M., Hall, V. and Morris, T. (2004). Outbreak of *Clostridium histolyticum* infections in injecting drug users in England and Scotland. *Euro. Surveill*. **9**.

Bryant, A., Stevens, D. and Tso, J. Effects of alpha toxin from *Clostridium perfringens* (Cp) on PMNL. *Proceedings and Abstracts of The American Society for Microbiology*, **77**. 1991.

Bryant, A.E., Awad, M.M., Lyristis, M., Rood, J.I. and Stevens, D.L. Perfringolysin O and phospholipase C from *Clostridium perfringens* impair host inflammatory cell defense mechanisms. *First International Conference on Molecular Biology and Pathogenesis of Clostridia*, Rio Rico, Arizona, **21**. 1995.

Bryant, A.E., Bayer, C.R., Hayes-Schroer, S.M. and Stevens, D.L. (2003). Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. *J. Infect. Dis*. **187,** 408–417.

Bryant, A.E., Bergstrom, R., Zimmerman, G.A., Salyer, J.L., Hill, H.R., Tweten, R.K., Sato, H. and Stevens, D.L. (1993). *Clostridium perfringens* invasiveness is enhanced by effects of theta toxin upon PMNL structure and function: The roles of leukocytotoxicity and expression of CD11/CD18 adherence glycoprotein. *FEMS Immunol. Med. Microbiol*. **7,** 321–336.

Bryant, A.E., Chen, R.Y.Z., Nagata, Y., Wang, Y., Lee, C.H., Finegold, S., Guth, P.H. and Stevens, D.L. (2000a). Clostridial gas gangrene II: Phospholipase C-induced activation of platelet gpIIb/IIIa mediates vascular occlusion and myonecrosis in *C. perfringens* gas gangrene. *J. Infect. Dis*. **182,** 808–815.

Bryant, A.E., Chen, R.Y.Z., Nagata, Y., Wang, Y., Lee, C.H., Finegold, S., Guth, P.H. and Stevens, D.L. (2000b). Clostridial gas gangrene I: Cellular and molecular mechanisms of microvascular dysfunction induced by exotoxins of *C. perfringens*. *J. Infect. Dis*. **182,** 799–807.

Bryant, A.E. and Stevens, D.L. (1996). Phospholipase C and perfringolysin O from *Clostridium perfringens* up-regulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce inter-

leukin-8 synthesis in cultured human umbilical vein endothelial cells. *Infect. Immun*. **64,** 358–362.

Bunting, M., Lorant, D.E., Bryant, A.E., Zimmerman, G.A., McIntyre, T.M., Stevens, D.L. and Prescott, S.M. (1997). Alpha toxin from *Clostridium perfringens* induces pro-inflammatory changes in endothelial cells. *J. Clin. Invest*. **100,** 565–574.

Centers for Disease Control and Prevention (2000). Update: *Clostridium novyi* and unexplained illness among injecting drug users—Scotland, Ireland, and England, April–June 2000. *MMWR* **49,** 543–545.

Farnell, M.B. (1987). Neutropenic enterocolitis: A surgical disease? *Infect. Surg*. **6,** 120–132.

Gorbach, S.L. (1992). *Clostridium perfringens* and Other Clostridia. In: *Infectious Diseases* eds. S.L. Gorbach, J.G. Bartlett and N.R. Blacklow, pp. 1587–1596. WB Saunders, Philadelphia.

Gordon, V., Benz, R., Fujii, K., Leppla, S. and Tweten, R. (1997). *Clostridium septicum* alpha-toxin is proteolytically activated by furin. *Infect. Immun*. **65,** 4130–4134.

Guerrant, R.L. (1990). Inflammatory Enteritides. In: *Principles and Practice of Infectious Diseases*, eds. G.L. Mandell, R.G. Douglas, and J.E. Bennett, New York: Churchill Livingstone, 870–880.

Houldsworth, S., Andrew, P.W. and Mitchell, T.J. (1994). Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect. Immun*. **62,** 1501–1503.

Johnson, S., Driks, M.R., Tweten, R.K., Ballard, J., Stevens, D.L., Anderson, D.J. and Janoff, E.N. (1994). Clinical courses of seven survivors of *Clostridium septicum* infection and their immunologic responses to α toxin. *Clin. Infect. Dis*. **19,** 761–764.

Jones, J.A. *et al*. (2002). An outbreak of serious illness and death among injecting drug users in England during 2000. *J. Med. Microbiol*. **51,** 978–984.

Kimura, A.C., Higa, J.I., Levin, R.M., Simpson, G., Vargas, Y. and Vugia, D.J. (2004). Outbreak of necrotizing fasciitis due to *Clostridium sordellii* among black tar heroin users. *Clin. Infect Dis*. **38,** e87–e91.

Law, D.A., Nannizzi-Alaimo, L. and Phillips, D.R. (1996). Outside-in integrin signal transduction: αIIbβ3-(gpIIbIIIa) tyrosine phosphorylation induced by platelet aggregation. *J. Biol. Chem*. **271,** 10811–10815.

Lawrence, G., Shann, F., Freestone, D.S. and Walker, P.D. (1979). Prevention of necrotizing enteritis in Papua, New Guinea by active immunization. *Lancet* **1,** 227–230.

Lawrence, G. and Walker, P.D. (1976). Pathogenesis of enteritis necroticans in Papua, New Guinea. *Lancet* **1,** 125–128.

Legat, F.J., Griesbacher, T. and Lembeck, F. (1994). Mediation by bradykinin of rat paw edema induced by collagenase from *Clostridium histolyticum*. *Br. J. Pharmacol*. **112,** 453–460.

Lipfert, L., Haimovich, B., Schaller, M.D., Cobb, B.S., Parsons, J.T. and Brugge, J.S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J. Cell Biol*. **119,** 905–912.

MacLennan, J.D. (1962). The histotoxic clostridial infections of man. *Bacteriol. Rev*. **26,** 177–276.

McGuigan, C.C., Penrice, G.M., Gruer, L., Ahmed, S., Goldberg, D., Black, M., Salmon, J.E. and Hood, J. (2002). Lethal outbreak of infection with *Clostridium novyi* type A and other spore-forming organisms in Scottish injecting drug users. *J. Med. Microbiol*. **51,** 971–977.

McNee, J.W. and Dunn, J.S. (1917). The method of spread of gas gangrene into living muscle. *Br. Med. J*. **1,** 727–729.

Noyes, H.E., Pritchard, W.L., Brinkley, F.B. and Mendelson, J.A. (1964). Analyses of wound exudates for clostridial toxins. *J. Bacteriol*. **87,** 623–629.

Ochi, S., Oda, M., Matsuda, H., Ikari, S. and Sakurai, J. (2004). *Clostridium perfringens* alpha-toxin activates the sphingomyelin metabolism system in sheep erythrocytes. *J. Biol. Chem*. **279,** 12181–12189.

Ohsaka, A., Tsuchiya, M., Oshio, C., Miyairi, M., Suzuki, K. and Yamakawa, Y. (1978). Aggregation of platelets in the mesenteric microcirculation of the rat induced by α toxin (phospholipase C) of *Clostridium perfringens. Toxicon* **16,** 333–341.

Regal, J.F. and Shigeman, F.E. (1980). The effect of phospholipase C on the responsiveness of cardiac receptors. I. Inhibition of the adrenergic inotropic response. *J. Pharmacol. Exp. Ther*. **214,** 282–290.

Robb-Smith, A.H.T. (1945). Tissue changes induced by *C. welchii* type A filtrates. *Lancet* **2,** 362–368.

Ruggeri, Z.M. (1997). Mechanisms initiating platelet thrombus formation. *Thromb. Haemostasis* **78,** 611–616.

Schirmer, J. and Aktories, K. (2004). Large clostridial cytotoxins: cellular biology of Rho/Ras-glucosylating toxins. *Biochim. Biophys. Acta* **1673,** 66–74.

Sellman, B.R., Kagan, B.L. and Tweten, R.K. (1997). Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. *Mol. Microbiol*. **23,** 551–558.

Smith, L.D.S. (1975b). Clostridial Wound Infections. In: *The Pathogenic Anaerobic Bacteria*, ed. L.D.S. Smith, Springfield, Il: Charles C. Thomas, 321–324.

Smith, L.D.S. (1975a). Clostridium. In: *The Pathogenic Anaerobic Bacteria*, ed. L.D.S. Smith, Springfield: Charles C. Thomas, 109–114.

Stevens, D.L. (1995). Clostridial Infections. In: *Atlas of Infectious Diseases,* ed. D.L. Stevens and G.L. Mandell, Philadelphia: Churchill Livingstone, 13.1–13.9.

Stevens, D.L. and Bryant, A.E. (1997). Pathogenesis of *Clostridium perfringens* infection: mechanisms and mediators of shock. *Clin. Infect. Dis*. **25,** S160–S164.

Stevens, D.L., Gibbons, A.E. and Bergstrom, R.A. Ultrastructural changes in human granulocytes induced by purified exotoxins from *Clostridium perfringens. Program & Abstracts of the American Society for Microbiology*. 1989.

Stevens, D.L., Mitten, J. and Henry, C. (1987). Effects of alpha and theta toxins from *Clostridium perfringens* on human polymorphonuclear leukocytes. *J. Infect. Dis*. **156,** 324–333.

Stevens, D.L., Musher, D.M., Watson, D.A., Eddy, H., Hamill, R.J., Gyorkey, F., Rosen, H. and Mader, J. (1990). Spontaneous, nontraumatic gangrene due to *Clostridium septicum. Rev. Infect. Dis*. **12(2),** 286–296.

Stevens, D.L., Titball, R.W., Jepson, M., Bayer, C.R., Hayes-Schroer, S.M. and Bryant, A.E. (2004). Immunization with the C-domain of alpha-toxin prevents lethal infection, localizes tissue injury, and promotes host response to challenge with *Clostridium perfringens. J. Infect Dis*. **190,** 767–773.

Stevens, D.L., Troyer, B.E., Merrick, D.T., Mitten, J.E. and Olson, R.D. (1988). Lethal effects and cardiovascular effects of purified alpha- and theta-toxins from *Clostridium perfringens. J. Infect. Dis*. **157,** 272–279.

Stevens, D.L., Tweten, R.K., Awad, M.M., Rood, J.I. and Bryant, A.E. (1997). Clostridial gas gangrene: Evidence that alpha and theta toxins differentially modulate the immune response and induce acute tissue necrosis. *J. Infect. Dis*. **176,** 189–195.

Sugahara, T., Takahashi, T., Yamaya S and Ohsaka, A. (1976). *In vitro* aggregation of platelets induced by alpha toxin (phospholipase C) of *Clostridium perfringens. Japan J. Med. Sci. Biol*. **29,** 255–263.

Tapley, P.M. and Murray, A.W. (1984). Platelet Ca2+-activated, phospholipid-dependent protein kinase: Evidence for proteolytic acti-

vation of the enzyme in cells treated with phospholipase C. *Biochem. Biophys. Res. Comm*. **118,** 835–841.

Whatley, R.E., Zimmerman, G.A., Stevens, D.L., Parker, C.J., McIntyre, T.M. and Prescott, S.M. (1989). The regulation of platelet-activating factor production in endothelial cells—the role of calcium and protein kinase C. *J. Biol. Chem*. **264,** 6325–6333.

Williamson, E.D. and Titball, R.W. (1993). A genetically engineered vaccine against alpha-toxin of *Clostridium perfringens* protects against experimental gas gangrene. *Vaccine* **11,** 1253–1258.

CHAPTER

# 56

# Staphylococcal exfoliative toxins

*Shamez N. Ladhani*

## INTRODUCTION

Staphylococcal skin infections remain one of the most common childhood conditions (Ladhani and Garbash, 2005). Of these, the staphylococcal scalded skin syndrome (SSSS) produces the most dramatic skin changes, with exfoliation of almost the entire body surface in severe cases (Ladhani and Evans, 1998; Ladhani *et al*., 1999a; Ladhani and Newson, 2000). While most cases are easily treated, it remains a potentially fatal condition, particularly in adults with underlying disease (Cribier *et al*., 1984). SSSS is caused by the staphylococcal exfoliative toxins, which have an exquisite ability to target and destroy desmoglein-1, a key component of the cytoskeleton in the epidermis (Ladhani, 2001; Ladhani, 2003). This results in disruption of the protective epidermal barrier, which presumably allows the organisms to proliferate and spread beneath this barrier (Amagai *et al*., 2000). At least four different exfoliative toxin serotypes (ETA, ETB, ETC, and ETD) are produced by *Staphylococcus aureus*, of which ETA, ETB, and possibly ETD produce disease in humans. At least six serotypes (SHETA, SHETB, ExhA, ExhB, ExhC, and ExhD) responsible for exudative dermatitis in piglets are produced by *Staphylococcus hyicus* (Sato *et al*., 2000; Ahrens and Andersen, 2004). Although the exfoliative toxins were discovered as far back as 1970, the mechanism by which exfoliation occurs has taken over 30 years to elucidate. This chapter will briefly summarize the clinical spectrum, histological features, risk factors, and the organisms responsible for SSSS and will then focus on the unique serine protease and superantigenic activities of the exfoliative toxins. Potential areas for future research, including development of new diagnostic tests, specific antitoxin therapies, and targeted drug delivery systems, are discussed at the end.

## CLINICAL FEATURES

SSSS was first described in 1878 by Baron Gotfried Ritter von Rittershain, a German physician who called the condition "dermatitis exfoliativa neonatorum" after observing almost 300 pediatric cases in a Czechoslovakian foundling asylum over a 10-year period (von Rittershain, 1878). The clinical features of SSSS vary from localized blisters to severe exfoliation affecting almost the entire body surface (Ladhani and Joannou, 2000). The localized forms of SSSS are also known as bullous impetigo, tropical bullous impetigo, measles pemphigoid, and bullous varicella (Lyell, 1979; Melish, 1982). Epidemiological studies on antitoxin antibodies suggest that, in the localized form, *S. aureus* enters the skin through a break in the skin barrier and produces the toxin locally, but hematogenous spread is limited by the presence of antitoxin antibodies (Melish *et al*., 1981), which may have been acquired from previous exposure to the toxins, for example, or, in young infants, from the mother through the placenta or during breastfeeding (Machida, 1995). Patients usually present with a cluster of characteristic fragile, thin-roofed, flaccid bullae, filled with fluid that varies from cloudy amber to frank pus (Melish, 1982). The surrounding skin remains normal, and there are no systemic symptoms or signs (Ginsburg, 1991). In neonates, the lesions are found mostly on the perineum, around the umbilicus, or both, while in older children, they occur mostly on the extremities (Melish, 1982).

In generalized SSSS (Figure 56.1), which is also known as Ritter's disease and pemphigus neonatorum in neonates, lack of protective host antibodies (Melish *et al*., 1981) allows widespread involvement of the entire skin surface with sparing of the mucous membranes (Melish *et al*., 1974; Pollack, 1996). Generalized SSSS can follow a localized staphylococcal infection of the skin, upper and lower respiratory tracts, the ears, conjunctiva, umbilical stumps, joints, and muscles (Melish, 1982; Wong *et al*., 1993; Bailey *et al*., 1995; Raymond *et al*., 1997), although in many cases, no primary focus is found (Cribier *et al*., 1984). Patients with generalized SSSS initially develop fever, malaise, lethargy, poor feeding, irritability, and a generalized, tender erythematous rash, which usually begins on the head and neck and spreads to the rest of the body over the next few days (Ochsendorf *et al*., 1988; Itani *et al*., 1992; Hoffmann *et al*., 1994). The rash is more marked in flexural creases, and the Nikolsky sign (gentle rubbing of uninvolved or healed skin produces dislodgement of the superficial epidermis) is usually positive. Soon after, large, fragile, thin-roofed blisters appear that rapidly rupture on the slightest pressure, resulting in large sheets of epidermis sloughing off to leave extensive areas of raw, denuded, varnish-like skin. In young infants, loss of the protective epidermis can result in poor temperature control, excessive fluid losses, and secondary infection, which can be fatal (Korting, 1994; Hoffmann *et al*., 1994). They may also develop staphylococcal septic shock and present with hypotension, tachycardia, neutropenia, and respiratory distress (Finch, 1988; Loughead, 1992).

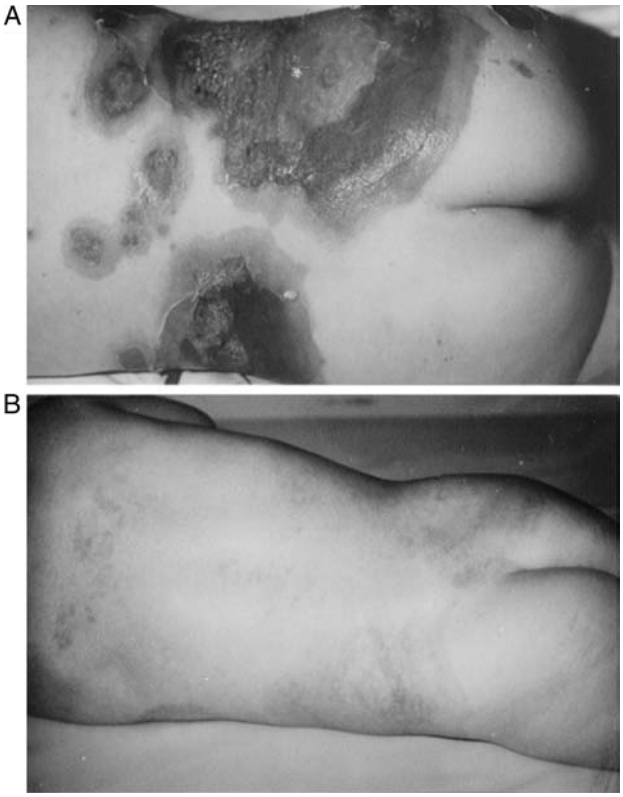



**FIGURE 56.1** (a) A two-year-old boy with generalized staphylococcal scalded skin syndrome. He presented with a 3-day history of fever and an erythematous rash, which evolved into well-demarcated superficial blisters that ruptured on slightest pressure to leave extensive areas of denuded skin. (b) The same boy one week after completing 5 days of intravenous antibiotic treatment. Note that the lesions have healed without scarring. (Reproduced from Ladhani (2001) and by permission of *Clinical Microbiology and Infection*, Blackwell-Synergy Publications)

## THE ORGANISM

SSSS is caused by *Staphylococcus aureus*, an aerobic, nonspore-forming, Gram-positive coccus belonging to the Micrococci family that is found in air, fomites, and dust, and commonly colonizes humans and animals (Lowy, 1998). It can be distinguished from other staphylococcal species on the basis of the yellow pigmentation it produces, β-hemolysis on blood agar, and positive results of coagulase, mannitol-fermentation, and deoxyribonuclease tests. *S. aureus* causes disease in two main ways—local invasion and toxin production. The organism produces a host of proteins and enzymes that are useful for tissue destruction and spread of infection—such as protease, lipase, and hyaluronidase—as well as protection against antibiotics, such as β-lactamases and penicillin-binding proteins. Like other skin commensals, *S. aureus* does not usually cause infection in healthy individuals (Darmstadt, 1997). However, when local barriers are broken, *S. aureus* will cause skin infections and may invade into deeper tissues to cause serious systemic illness (Ladhani and Garbash, 2005). Skin and soft tissue infections are often described as either primary or secondary. Primary infections involve apparently healthy skin with no obvious site of skin break to explain the lesion, while secondary infections occur in diseased skin, such as atopic dermatitis or in previously healthy skin that has been traumatized by, for example, surgery, burns, or bites. *S. aureus* is the most common cause of skin and soft tissue infections, accounting for over half of all organisms isolated in large epidemiological studies (Darmstadt, 1997; Doern *et al*., 1999; Jones *et al*., 1999; Gales *et al*., 2000; Laupland *et al*., 2003). *S. aureus* is responsible for folliculitis, furunculosis, carbunculosis, and cellulitis (Failla and Pankey, 1994; Rhody, 2000; Sharma and Verma, 2001; Chiller *et al*., 2001; Stulberg *et al*., 2002; Mengesha and Bennett, 2002), as well as surgical and traumatic wound infections,

mastitis, and neonatal oomphalitis. Other skin and soft tissue infections may also be caused by *S. aureus*, but are often polymicrobial in origin. These include burns, decubitus ulcers, puncture wounds of the foot where *Pseudomonas aeruginosa* is often implicated, and mammalian bites, which are often co-infected with other multiple antibiotic–resistant organisms (Griego *et al*., 1995). In addition to local invasion, *S. aureus* can cause disease by production of exotoxins, such as enterotoxins, which cause gastroenteritis; toxic shock syndrome toxin-1, which causes the toxic shock syndrome; and the exfoliative toxins, which are responsible for the bullous impetigo and SSSS.

## Staphylococcal virulence factors

Like other major pathogens, *S. aureus* is adapted to identify changes in its environmental conditions and regulate expression of different factors that alter colonization, multiplication, invasion, spread, destruction of host immune defenses, and cause multi-organ failure (Archer, 1998). The pathogenicity of *S. aureus* is multifactorial and depends on a wide range of cell wall–associated (e.g., capsular polysaccharide, Protein A, and fibronectin binding protein) and extracellular virulence factors (e.g., coagulase, hemolysins, enterotoxins, epidermolysins, and synergohymenotropic toxins) produced by the organism (Novick, 2000). The *agr* gene locus controls the expression of most of the virulence factors (including the exfoliative toxins) by increasing the production of extracellular proteins and suppressing the expression of cell surface proteins (Ji *et al*., 1995; Jarraud *et al*., 2003). This locus encodes a two-component signaling pathway whose activating ligand is a bacterial density-sensing, auto-inducing octapeptide that is itself also encoded by the *agr* locus. Four major polymorphisms in the amino acid sequence of either the autoinducing peptide or its corresponding receptor have been described and termed *agr* I–IV, and some polymorphisms may be associated with a specific staphylococcal infection. Generalized exfoliation in the staphylococcal scalded skin syndrome, for example, is associated with *agr* group IV, while toxic shock syndrome is associated with *agr* group III (Ji *et al*., 1997; Jarraud *et al*., 2003).

*S. aureus* produces a range of different virulence factors that are associated with skin and soft tissue infections. One such virulence factor, known as epidermal cell differentiation inhibitor (EDIN), is associated with the exfoliative toxins and SSSS. At least three isoforms of staphylococcal EDIN, which are so called because EDIN-A was first discovered as an inhibitor of morphological differentiation of epidermal keratinocytes *in vitro*, have been reported (Sugai *et al*., 1992). The EDINs belong to the C3 family of bacterial Rho-specific mono-ADP-ribosyltransferases that specifically ribosylate and inactivate small eukaryotic GTPases belonging to the Rho family, which play a vital role in regulating the eukaryotic actin cytoskeleton (Sugai et al., 1992; Yamaguchi *et al*., 2001). The gene for ETB is located within the same pathogenicity island as EDIN-C (Yamaguchi *et al*., 2001), while ETD was discovered in a pathogenicity island that also encodes for EDIN-B (Yamaguchi *et al*., 2002). Pathogenicity islands are mobile DNA segments of variable sizes that encode various virulence genes in over 30 different bacterial species and can be transferred horizontally (Hacker *et al*., 2003; Novick, 2003; Schmidt and Hansel, 2004). LukE-LukD is another virulence factor that is produced by over 75% of *S. aureus* isolates from cases of impetigo, compared to less than a third of isolates from other primary skin infections, routine hospital isolates, or nasal carriers (Gravet *et al*., 2001). LukE-LukD is staphylococcal pore-forming leukotoxin that can induce dermonecrosis in rabbits (Gravet *et al*., 1998). The clinical significance of the association between the exfoliative toxins and such virulence factors and their role in the pathogenesis of SSSS is not clear and warrants further research.

## Epidemiology and risk factors for staphylococcal infections

*S. aureus* remains one of the most important causes of life-threatening bacterial infections in humans (Lowy, 1998; Laupland *et al*., 2004). Large epidemiological studies consistently identify *S. aureus* among the top five causes of bacteremia in all age groups (Reacher *et al*., 2000). Despite advances in the prevention and management of infections, the incidence of both community-acquired and hospital-acquired staphylococcal infections over the past 20 years continues to rise, with little change in mortality rates (Pittet and Wenzel, 1995; Ayliffe 1997; Kallman *et al*., 1997; Archer, 1998; Reacher *et al*., 2000; Laupland *et al*., 2003). This rise parallels more widespread use of invasive procedures, increasing severity of illness in hospitalized patients, poor compliance with infection control practices, increasing selection of antibiotic-resistant strains, intravenous drug abuse, and an increasing prevalence of diabetes (Lowy, 1998; Laupland *et al*., 2003). In the pre-war era, mortality due to invasive *S. aureus* infection was reported to be over 80% and, although the discovery of penicillin and other antibiotics resulted in a dramatic reduction in mortality, it still remains around 25%, with reported mortality rates of 11–43% over the past 25 years (Mylotte *et al*., 1987; Archer, 1998; Laupland *et al*., 2003). People who are colonized with *S. aureus* are at

higher risk for subsequent development of staphylococcal infections (Weinke *et al*., 1992; Wenzel and Perl, 1995; Weinstein, 1995; Kluytmans *et al*., 1995; von Eiff *et al*., 2001; Huang and Platt, 2003). Other predisposing factors for developing staphylococcal infections include immunodeficiency (including HIV), diabetes mellitus, chronic granulomatous disease, defects in the mucocutaneous barriers produced by trauma including surgery, foreign surfaces (sutures, shunts, intravascular catheters), burns, as well as frequent and/or multiple hospital admissions (Drews *et al*., 1995; Raymond and Aujard, 2000; Hudome and Fisher, 2001; Grohskopf *et al*., 2002).

### Commensal carriers of *S. aureus*

Humans are a natural reservoir of *S. aureus*, with 25–50% of the general population being nasal carriers and 10–20% being persistently colonized (Noble *et al*., 1967; Ladhani *et al*., 1999b). Individuals with primary skin disorders (such as atopic dermatitis, contact dermatitis, and psoriasis), underlying diseases (such as diabetes mellitus and acquired immunodeficiency syndrome), those undergoing hemodialysis, and surgical patients, have higher staphylococcal carriage rates of up to 100% (Lowy, 1998; Abeck and Mempel, 1998; Noble, 1998; Hussain *et al*., 2001; Nakamura *et al*., 2002; Arkwright *et al*., 2002). In neonates, *S. aureus* is also commonly found on the skin around the eyes, perineum, wound sites, circumcision wounds, and umbilical stumps (Annunziato and Goldblum, 1978; Opal *et al*., 1988; Dancer *et al*., 1988; Peacock *et al*., 2003). Inanimate objects such as stethoscope bells, ophthalmoscope and otoscope handles, laundry carts, nursery magazines, and ventilation shafts in hospitals may also harbor *S. aureus* and can be responsible for prolonged epidemics of staphylococcal infections in neonatal nurseries (Kaplan *et al*., 1986).

### Exfoliative toxin-producing *S. aureus*

The association between *S. aureus* and SSSS was made as far back as 1891 when Almquist isolated the organism from a patient with pemphigus neonatorum and initially called it *Micrococcus pemphigi neonatorum* (Almquist, 1891). In 1956, Lyell described a skin condition in adults and children that resembled scalded skin and called it toxic epidermal necrolysis (Lyell, 1956). He did not realize that he had described two unrelated conditions until 1967 when he reviewed 128 cases that fitted his diagnostic criteria for toxic epidermal necrolysis and found that almost a quarter—all children under 10 years of age—presented predominantly with staphylococcal infection (Lyell, 1967). Subsequent studies showed that the skin lesions associated with *S. aureus* infection in children occurred specifically within the midepidermal layer of the skin, while in adults the skin lesions were not associated with staphylococcal infection and occurred at the dermoepidermal junction (Lowney *et al*., 1967; Lyell *et al*., 1969; Lyell, 1979).

In Europe and the United States, most *S. aureus* strains responsible for SSSS belong to phage group II, particularly types 71 and 55/71 (Parker *et al*., 1955; Melish and Glasgow, 1970; Dajani, 1972), which account for 7–25% of human *S. aureus* isolates in the United Kingdom (Dancer and Noble 1991). Around 30–40% of phage group II *S. aureus* strains produce exfoliative toxin (Kapral, 1975; Fleurette and Ritter, 1980; Willard *et al*., 1982). In Japan, most strains causing SSSS belong to phage groups other than II (Kondo *et al*., 1974; Sarai *et al*., 1977; Murono *et al*., 1988), and one Nigerian study reported that almost 60% of toxin-producing staphylococcal strains were non-typeable, with phage group II strains accounting for only 18% (Adesiyun *et al*., 1991).

The lack of a suitable and standardized assay for the exfoliative toxins has meant that there is very little literature on the epidemiology of SSSS. One prospective study of 944 *S. aureus* isolates from 577 dermatological patients of all age groups reported a 5.1% carriage rate of exfoliative toxin-producing *S. aureus*, although only 32% of these patients presented with clinical features of SSSS, indicating that a large proportion were simply asymptomatic carriers (Elsner and Hartman, 1988). Another epidemiological survey revealed that 33% of 500 pregnant women attending an antenatal clinic carried at least one strain of *S. aureus* in the nose, axilla, and/or perineum and, of the 184 *S. aureus* isolates, 5 (2.7%) produced exfoliative toxin (Dancer and Noble, 1991). Other studies have reported toxin production in 6% of 2,632 *S. aureus* isolates from hospitalized adults and children (Piemont and Monteil, 1983), 4.4% of 194 isolates from human diarrhea and wounds (Adesiyun *et al*., 1991), 19% of 100 isolates from the mouths of children with dental disease (Miyake *et al*., 1991), and 40% of isolates from superficial skin infections (Arbuthnott, 1981). One Nigerian study reported that 3.9% of 666 *S. aureus* isolates from healthy animals also produced exfoliative toxin; however, their importance as reservoirs for human SSSS is not known (Adesiyun *et al*., 1991).

## THE EXFOLIATIVE TOXINS

Although the presence of a soluble toxin in the pathogenesis of SSSS was suspected as far back as the 1950s (Lyell, 1956), the first clue of its existence came only

after Melish and Glasgow (1970) showed that sterile fluid obtained from intact bullae and sterile supernatant from phage group II *S. aureus* cultures were able to reproduce the skin lesions of SSSS, with a positive Nikolsky sign in neonatal mice. Even intraperitoneal inoculation resulted in exfoliation, indicating that the toxin had its specific target in the skin. Within a year, the active component in the supernatant was isolated, purified, characterized, and termed exfoliatin or epidermolysin (Kapral and Miller, 1971; Melish *et al*., 1972; Arbuthnott *et al*., 1971). Soon after, it was realized that at least two different toxin serotypes existed (Kondo *et al*., 1974). The second serotype was isolated and characterized as a heat-labile toxin with a similar molecular weight to the original toxin. This second serotype, termed ETB, produced identical clinical and histological lesions in humans and newborn mice as the original serotype, ETA (Kondo *et al*., 1975b). Both toxins were soon isolated, sequenced, characterized, and cloned into other bacteria such as *Escherichia coli* (Kapral and Miller, 1971; Kondo *et al*., 1975a; Jackson and Iandolo, 1986; Sakurai *et al*., 1987; Ladhani *et al*., 2002). ETA consists of 242 amino acids, has a molecular mass of 26,950 Da, and is heat stable, while ETB consists of 246 amino acids, has a molecular mass of 27,274 Da, and is heat labile (Kondo *et al*., 1975a; Bailey *et al*., 1980). Like many other staphylococcal virulence factors, the toxins are encoded in pathogenic islands, which are capable of horizontal transfer between bacteria (Novick, 2003). The *eta* gene is chromosomally located within a 43 kb temperate phage ϕETA that is capable of converting a toxin-negative *S. aureus* strain into an ETA-producer, while the *etb* gene is located on a 38.2–38.5 kb plasmid (Lee *et al*., 1987; Yamaguchi *et al*., 2000). In the United Kingdom and Ireland, 32% of 116 *S. aureus* isolates from patients with suspected SSSS produced ETA, 27% produced both ETA and ETB, and 12% produced ETB only, with neither toxin being detected in 34 strains (De Azavedo and Arbuthnott, 1981). A large epidemiological study in Germany found 98% of the exfoliative toxin–producing isolates from dermatological patients produced ETA only (Elsner and Hartman, 1988). In France, most cases of childhood SSSS were associated with ETA alone (89%) with only a few cases due to both ETA and ETB (8%) or ETB alone (4%) (Piémont and Monteil, 1983; Willard *et al*., 1982). Similarly, a Nigerian study reported that ETA accounted for 91.1% of the 34 toxin-producing strains isolated from a range of human and animal sources (Adesiyun *et al*., 1991). In the United States, 40% of *S. aureus* isolates from SSSS patients produced ETA, 36% produced both, and 16% produced ETB only (Melish, 1982). On the other hand, ETB is more prevalent in Japan, with two studies reporting ETB, ETA, and ETB, and ETA production rates of 52, 23, and 25% of 61 isolates (Murono *et al*., 1988) and 35, 37, and 21% of 43 isolates from patients with SSSS (Kondo *et al*., 1975b), respectively.

At least two other antigenically distinct toxin serotypes produced by *S. aureus* have been identified more recently (Ladhani *et al*., 2003). ETC, which was isolated from a horse with skin infection and phlegmon, is a 27-kDa heat-labile toxin that produces intraepidermal splitting in both newborn mice and chicks (Sato *et al*., 1994). The role of ETC in human SSSS is not known. Recently, another 28-kDa serotype, termed ETD, with 40% sequence similarity to ETA, 59% to ETB, and 13% to ETC, was identified while screening the genome of clinical *S. aureus* isolates with probes for the *eta* and *etb* genes (Yamaguchi *et al*., 2002). ETD produces exfoliation in newborn mice but not in one-day-old chicks. Intriguingly, two of the three ETD-producing strains identified in this study were isolated from wound infections and not SSSS. In a parallel study of Japanese patients with impetigo, only one of 88 *S. aureus* isolates produced ETD (Yamaguchi *et al*., 2002). The authors speculate that ETD may have a wider pathogenic role in staphylococcal infections, such as disrupting the skin epithelial barrier and allowing the organism to spread or invade local tissues.

An extensive search for similar toxins among 461 human clinical isolates of 18 different staphylococcal species other than *S. aureus* using multiplex polymerase chain reaction did not identify any toxin genes (Becker *et al*., 2001). However, in piglets, certain strains of *S. hyicus* are able to produce exotoxins that are responsible for exudative epidermitis, a skin disease characterized by exudation, exfoliation, and blister formation with skin erosions (Tanabe *et al*., 1993). They are able to produce midepidermal cleavage in less than five-week-old piglets and one-day-old chicks, but not older chicks, mice, or guinea pigs (Tanabe *et al*., 1993; Anderson, 1998). Two toxin serotypes, designated SHETA and SHETB (Sato *et al*., 2000), have been isolated in Japan and four others, termed ExhA, ExhB, ExhC, and ExhD, have been isolated in Denmark (Ahrens and Andersen, 2004). The molecular sizes of the protoxins are approximately 30 kDa, while the mature toxins are 27 kDa in size and 816–834 bases in length (Ahrens and Andersen, 2004). They have a 45–70% homology to the *S. aureus* exfoliative toxins at DNA level and 49–54% homology at the protein level. There is no species-specific clustering of the amino acid sequences. Instead, ExhC is more similar to ETA than other *S. hyicus* exfoliative toxins, while SHETB has a higher similarity to ETD (Ahrens and Andersen, 2004). Interestingly, the aspartate residue constituting the active site of all serine proteases is replaced with glutamate in ExhD. However, this substitution is unlikely to

change the biochemical properties of the toxin, and histologically, the skin lesions produced by ExhD are identical to the other exfoliative toxins (Ahrens and Andersen, 2004).

## Risk factors for developing SSSS

SSSS is predominantly a disease of infants and young children. Initial animal studies that led to the discovery of the toxins showed that mice younger than five days old developed exfoliation after inoculation with a toxigenic *S. aureus* strain, while those over seven days old did not (Melish and Glasgow, 1970). Similar observations were made in baby chicks, piglets, and other animals (Ladhani *et al*., 2003), suggesting that there may be a specific maturation process that protects adults from developing generalized SSSS. However, anatomical differences in the skin are unlikely to explain this observation because immunosuppressed adults and those with renal failure can also develop generalized SSSS (Cribier *et al*., 1984). SSSS has also been reported in healthy, immunocompetent young adults (Opal *et al*., 1988), and intradermal injection of purified toxin into the forearm of a healthy adult will result in mid-epidermal splitting and blister formation (Wiley *et al*., 1974).

On the other hand, there is increasing evidence to show that renal function plays a critical role in determining susceptibility to generalized SSSS. An extensive literature review observed that over 75% of adults who develop generalized SSSS had impaired renal function (Cribier *et al*., 1984). Animal studies have shown that newborn mice excrete only one-fifteenth of a test dose of intravenous exfoliative toxin within three hours, compared to a third of the test dose by adult mice (Fritsch *et al*., 1976). Furthermore, nephrectomized, but not hepatectomized, adult mice will develop exfoliation when challenged with exfoliative toxin (Fritsch *et al*., 1976). Another recent study demonstrated that serum toxin levels peaked at 120 minutes after injection of a test dose and were still detectable in significant amounts at 16 hours in one-day-old mice. In 10-day-old mice, however, toxin levels peaked at 60 minutes and then dropped sharply to undetectable amounts by 6 hours—exfoliation was noted in the one-day-old mice at 2–4 hours post-injection but not in the 10-day-old mice (Plano *et al*., 2001). Furthermore, repeated hourly injections to maintain a maximal level of toxin for a sustained period of time resulted in exfoliation in 10-day-old and 21-day-old adult mice, suggesting that a critical amount of toxin is required in the epidermis to induce exfoliation. These findings suggest that poor renal clearance of exfoliative toxin in neonates and young children results in high levels of toxin in the bloodstream, thereby allowing a critical amount of the toxin to reach the epidermis and induce exfoliation. In adults, this may occur when patients are critically ill, immunocompromized, or have renal failure. However, other factors are likely to be important since only a small proportion of patients with renal failure develop generalized SSSS.

Immune function has also been considered to be an important risk factor for developing SSSS. One animal study showed that immunization with purified extracts of exfoliative toxin protected neonatal mice from exfoliation when inoculated with toxigenic *S. aureus* strains (Melish *et al*., 1981), while another showed that passive transfer of maternal antitoxin antibodies played an important role in protecting newborns from exfoliation following subcutaneous innoculation of exfoliative toxin (Machida, 1995). In humans, a significant proportion of adults who develop SSSS, for example, are immunocompromized (Cribier *et al*., 1984). Antibody studies have shown that 13 of 21 children (62%) with localized SSSS had antitoxin antibodies in the acute phase (within five days) of the infection and 100% in the convalescent phase (14 days after infection) (Melish *et al*., 1981). In contrast, none of the 10 children with generalized SSSS had antitoxin antibodies in the acute phase compared to 100% in the convalescent phase (Melish *et al*., 1981). However, a recent animal study reported that adult mice that were thymectomized at birth (and, therefore, lacking mature T cells) and adult mice with severe combined immunodeficiency (SCID) lacking both mature T and B cells (and, therefore, not able to produce protective antibodies) were not susceptible to exfoliation (defined as a positive Nikolsky sign and/or blister formation at 16 hours after subcutaneous toxin injection) (Plano *et al*., 2001). Furthermore, injecting adult spleen cells into neonatal mice did not protect against exfoliation when challenged with exfoliative toxin, suggesting that the adaptive immune response, which develops after the first week of life in mice, does not play a significant role in protection against exfoliation (Plano *et al*., 2001). Further studies are, therefore, required to elucidate the role of the host immune system in developing SSSS.

## Mechanism of action of the exfoliative toxins

In generalized SSSS, the exfoliative toxins are made during the post-exponential phase of bacterial growth (Bailey *et al*., 1995) and excreted from colonizing staphylococci before being absorbed into the systemic circulation (Melish *et al*., 1981). The toxins reach the zona granulosa of the epidermis by diffusing through dermal capillaries. Injection of $^{125}$Iodine-labeled toxin into neonatal mice has shown that, apart from the skin,

there is no significant binding of the toxins to any other body organ (Fritsch *et al*., 1976). The toxins are species specific—ETA and ETB, for example, only affect humans, monkeys, mice, and hamsters, but not rats, rabbits, voles, chickens, guinea pigs, or frogs (Kapral and Miller, 1971; Bailey *et al*., 1995; Elias *et al*., 1975). The lesions of localized and generalized SSSS are histologically identical, except that *S. aureus* is sometimes seen in localized forms of SSSS but rarely in generalized SSSS (Ladhani *et al*., 1998). *In vitro* studies have shown that addition of exfoliative toxin to human keratinocyte cultures obtained from plastic surgery or mouse organotypic skin cultures results in the disappearance of small vesicles that are usually present between the cells and formation of intercellular fluid-filled gaps between the zona granulosa and zona spinosum of the epidermis, followed by appearance of midepidermal splitting, which is characteristic of SSSS (Lillibridge *et al*., 1972; McCallum, 1972). There is no inflammatory response, cytolysis, cell degeneration, or necrolysis (Dedobbeleer and Achten, 1975; Elias *et al*., 1975; Wuepper *et al*., 1975; Hoffmann *et al*., 1994).

Despite discovery of the exfoliative toxins as the cause of SSSS in the early 1970s, it took another 30 years to identify the mechanism by which the toxins caused midepidermal splitting. The toxins do not have any significant *in vitro* enzymatic activity towards a wide range of non-specific substrates tested (Arbuthnott *et al*., 1974; Rogolsky *et al*., 1974; Kondo, 1976; Bailey *et al*., 1980; Wiley and Rogolsky, 1985), and exfoliation in newborn mice is not significantly affected by any of a wide range of metabolic inhibitors (Smith and Bailey, 1990; Bailey *et al*., 1995). Initial studies suggested that the toxins may have intrinsic lipase activity because a significant proportion of *S. aureus* isolates from patients with SSSS had lipolytic activity (Arbuthnott *et al*., 1969; Tait, 1973). In addition, incubation of purified toxin with egg yolk emulsion or bovine sphingomyelin resulted in a dose-dependent release of phosphate, suggesting that the toxin had intrinsic phospholipase activity (Wiley and Rogolsky, 1985), although other investigators were unable to detect any lipase activity towards triacylglycerol or phospholipids (Prevost *et al*., 1991).

The idea that the exfoliative toxins may act as atypical serine proteases came when sequence analysis studies showed that the exfoliative toxins had a 25% primary sequence homology with staphylococcal V8 protease (26% with ETA and 31% with ETB), a member of the trypsin-like serine protease family that does not possess any disulfide bridges and has an unusual specificity for cleaving the carboxyl-terminal side of acidic amino acid residues (Drapeau, 1978). Furthermore, the highly conserved serine-histidine-aspartate catalytic triad that forms the active site of all serine proteases was also present in the exfoliative toxins (Dancer *et al*., 1990). Mutation of any of the three amino acids forming the catalytic triad resulted in loss of exfoliative activity when injected into newborn mice (Prevost *et al*., 1991; Redpath *et al*., 1991). In addition, phenylmethylsulfonyl fluoride (PMSF), a specific serine protease inhibitor, delayed exfoliation by ETA in neonatal mice (Dancer *et al*., 1990). At around the same time, Bailey and Redpath (1992) tried to determine whether the toxins might have an esterolytic activity on the basis that proteases are always esterases and serine proteases tend to have a higher catalytic efficiency toward esters (Ferscht, 1985). They demonstrated that both ETA and ETB have very low but significant *N-t*-butoxycarbonyl L-glutamic acid α-phenyl (boc-L-Glu-*O*-phenyl) esterase activity, which was not present in a mutant form of ETA where the Ser-195 of the putative catalytic site was replaced by glycine (Bailey and Redpath, 1992). Furthermore, the pH dependence of both ETA and ETB in this reaction was typical of the histidine-associated $pK_a$ found with all serine proteases.

Takiuchi and colleagues (1987) initially showed that neonatal mouse epidermis incubated with exfoliative toxin at 37°C for 12 h produced a four-fold increase in caseinolytic activity in the supernatant after epidermal cells were removed by centrifuging at 1,000 *g* for 15 min, when compared to epidermis alone, ETA alone, or ETA boiled for 40 min at 100°C before incubation with epidermis. This activity was inhibited by $\alpha_2$-macroglobulin, indicating the activation of proteases or inhibition of protease inhibitors in the supernatant. Protease activity in the supernatant was also reported when exfoliative toxin was incubated for 20 hours with neonatal mouse epidermal extracts, formed by grinding neonatal mouse epidermis in liquid nitrogen, followed by suspension in buffer and high-speed centrifugation (Jaulhac *et al*., 1994). Incubation of exfoliative toxins with A431 cells (a human squamous cell line) was also shown to induce proteolytic activity in the supernatant, which could be abolished by pre-incubation with the specific serine protease inhibitor, PMSF (Ladhani *et al*., 1999b). A 20-kDa protease was subsequently reported to be responsible for the casein hydrolyzing activity in the supernatant (Ninomiya *et al*., 2000). This protease produced identical skin lesions when injected subcutaneously into newborn mice and cross-reacted with anti-exfoliative toxin antibodies, leading the authors to speculate that the exfoliative toxins were pro-enzymes and interaction with an epidermal component changed the structure or size of the toxins and converted them into active proteases.

A major step forward in our understanding of the toxins' possible mechanism of action came after

Barbosa and colleagues (1996) used the structures of known glutamate-specific, trypsin-like serine proteases to develop a computer model of the three-dimensional structure of ETA (Figure 56.2). They predicted that the toxins consist of two domains, each consisting of six-strand β-barrels common to all members of the trypsin family; an active site, formed by the serine-histidine-aspartate catalytic triad that is located on the interface of the two barrels; and a C-terminal α-helix, although the toxin had a larger N-terminal portion than the other members. The active site, formed by the serine-histidine-aspartate catalytic triad, is located on the interface of the two barrels and a C-terminal α-helix. The toxins additionally possess threonine-190 and histidine-213 (chymotrypsin numbering) in the core of the N-terminal S1 pocket that are conserved in all glutamate-specific serine proteases (Vath *et al*., 1997; Cavarelli *et al*., 1997). Homology to the *Streptomyces griseus* protease Glu-SGP suggested that ETA and ETB cleaved after a glutamic acid (or possibly aspartic acid) residue that would be stabilized in the active site by histidine-213, threonine-190, and lysine-216. Unlike other members of the trypsin family, however, the exfoliative toxins have a distinct, highly-charged (8 of 15 residues in ETA and 6 of 11 residues in ETB) N-terminal α-helix near the base of the S1 pocket. Furthermore, the proline-192-glycine-193 bond in ETA and its equivalent valine-183-glycine-184 in ETB are flipped 180° relative to other serine proteases. This allows the proline-192 in ETA and valine-183 in ETB to form a hydrogen bond with their respective active site serine residue, thus blocking the active site. This would explain why the toxins are inactive in their native state. Their computer predictions were soon confirmed by crystallographic data for both ETA and ETB (Vath *et al*., 1997; Cavarelli *et al*., 1997; Vath *et al*., 1999).

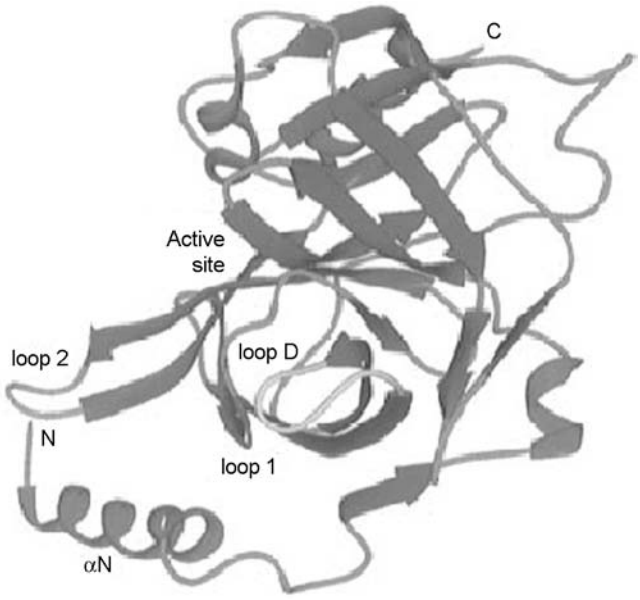



**FIGURE 56.2** Ribbon diagram of the structure of ETA. Like other members of the chymotrypsin-like family, ETA consists of two perpendicular domains, each made up of six antiparallel β-strands that form a β-barrel, and a carboxy-terminal helix. The active site, formed by the serine-histidine-aspartate catalytic triad, is located on the interface of the two barrels and the carboxy-terminal helix. However, the amino-terminal domain of ETA has a unique, highly charged, extended amphipathic 15-residue α-helix (αN) containing a four-turn helical loop. The αN helix is in contact with both loops 1 and 2 of the carboxy-terminal and prevents loop 2 from folding into the conformation seen in other serine proteases. Loop 2 is considered to be important for the proper positioning of substrate in the active site. It has been proposed that binding of the αN helix to a specific region on desmoglein-1 results in a conformational change that exposes the active site of the toxin, which can then bind and cleave the protein. (Reproduced from Vath *et al.* (1997) and by permission of *Biochemistry*, American Chemical Society)

It has been proposed that binding of the highly charged N-terminal α-helix to a specific epidermal receptor could flip the proline-glycine bond in ETA (and valine-glycine in ETB) to allow a conformational change that exposes the active site, which can then bind and cleave the substrate (Vath *et al*., 1997; Cavarelli *et al*., 1997; Vath *et al*., 1999). Free energy calculations suggest that it would require less than 3 kCal/mol of energy to cause this flip, and there are no steric hindrances to prevent the flip from occurring (Cavarelli *et al*., 1997). A similar mechanism has been identified in other proteases, including thrombin and hepatitis C virus NS3 protease (Cavarelli *et al*., 1997). Interestingly, the catalytic triad that forms the active site is not conserved in *S. aureus* ETC, *S. hyicus* exfoliative toxin A (SHETA), or the recently cloned exfoliative toxin from *S. intermedius*, although they are able to produce identical skin lesions in their respective animal models (Yamaguchi *et al*., 2002; Terauchi *et al*., 2003).

The target for the exfoliative toxins in the epidermis has only been identified recently. Previous studies had suggested that gangliosides may be the epidermal receptors for the exfoliative toxins (Sakurai and Kondo, 1979). Pre-incubation of ETA with a GM4-like—but not GM1-, GM2-, or GM3-like—glycolipid extract from the skin of one-day-old suckling mice for three hours resulted in loss of exfoliative activity when subcutaneously injected into neonatal mice, suggesting that the GM4-like glycolipids present in neonatal but not adult mice were possible receptors for the toxins and would account for the age and species specificity of the toxins (Sakurai and Kondo, 1979; Tanabe *et al*., 1995). On the other hand, another group showed that both ETA and ETB could cleave alpha- and beta-melanocyte-stimulating hormones, and this activity was not present with biologically inactive mutants of

the toxins, suggesting that these proteins may be the target for the toxins in the epidermis (Rago *et al*., 2000).

However, in a series of elegant experiments, the true epidermal target for the exfoliative toxins was identified following observations by Amagai and colleagues (2000) that the skin lesions of SSSS were clinically and histologically identical to pemphigus foliaceus (Figure 56.3), an autoimmune skin condition in which autoantibodies attack desmoglein-1, a transmembrane desmosomal glycoprotein in the cadherin gene superfamily, which plays an important role in maintaining keratinocyte cell-to-cell adhesion in the superficial epidermis (Angst *et al*., 2001). The group showed that injecting small quantities of purified exfoliative toxin into the neck of newborn mice produced identical blisters to pemphigus foliaceus, and using immunofluoresence staining and Western blot analysis, they were able to demonstrate that the blister formation was specifically due to degradation of desmoglein-1, with no reaction occurring when ETA was incubated with the closely related desomglein-3 or E-cadherin (Amagai *et al*., 2000; Hanakawa *et al*., 2002). Desmoglein-1 is found throughout the epidermis and mucous membranes, but blisters are only formed in the superficial epidermis in both SSSS and pemphigus foliaceus because desmoglein-3, which is found in the deep epidermis and in mucous membranes but not in the superficial epidermis, will compensate for desmoglein-1 and maintain the integrity of the structure if the function of the latter is compromised (the "desmosome compensation" hypothesis). This explains why the mucous membrane is not affected in SSSS (Mahoney *et al*., 1999).

Using wild-type ETA and mutant ETA that is able to bind but unable to cleave desmoglein-1, the dissociation constant of the reaction between ETA and desmoglein-1 has been estimated at 8μm, which is within the range of a hydrolytic reaction whose specificity is due to relatively strong binding in the catalytic site with a relatively low Km (Hanakawa *et al*., 2002). Co-immunoprecipitation studies between ETA and desmoglein-1 and between ETA and other closely related epidermal antigens, such as desmoglein-3 and E-cadherin, also confirm that ETA binds specifically to

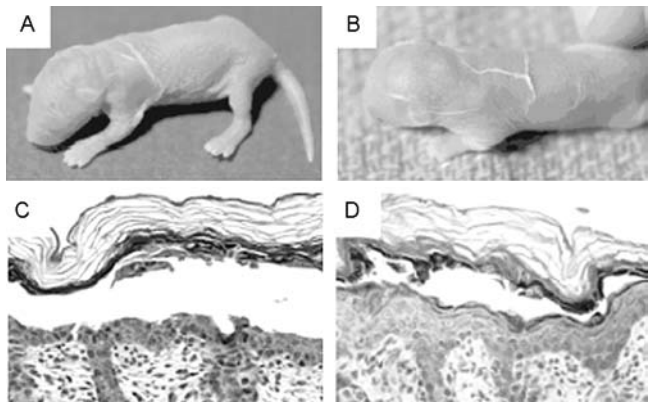



**FIGURE 56.3** Neonatal mice aged 1-2 days injected with pemphigus foliaceus autoantibodies against desmoglein-1 (a) and exfoliative toxin (b) showed identical superficial blistering and exfoliation. The histological appearance of the blisters showing midepidermal cleavage is also identical with both the antibodies (c) and exfoliative toxin (d). (Reproduced from Amagai *et al.* (2000) and by permission of *Nature Medicine*, Nature Publishing Group)

desmoglein-1 and dissociates after hydrolysis of the target peptide bond, consistent with enzyme recycling (Hanakawa et al., 2002). Kinetic studies show that the values for $K_{cat}/K_m$, which is a measure of enzyme efficiency, of $6.2 \times 10^4\ M^{-1}\ s^{-1}$ for ETA, $2.6 \times 10^4\ M^{-1}\ s^{-1}$ for ETB, and $1.9 \times 10^4\ M^{-1}\ s^{-1}$ for ETD, suggest very efficient proteolysis of a large substrate by the toxins (Hanakawa *et al*., 2004). These values are similar to those for the acidic amino acid-specific *S. aureus* V8 protease and glutamic acid-specific *Streptomyces griseus* proteinase (Hanakawa *et al*., 2004).

Based on the crystal structure of other cadherins, desmoglein-1 is predicted to form a rod-like structure with four highly conserved, homologous amino-terminal extracellular domains (EC1-4) of about 100 amino acids each that are stabilized and orientated by calcium-binding sites between these repeats, and a fifth extracellular region (EC5) intervenes between the repeats and the plasma membrane and is more variable in size and sequence (Figure 56.4). The primary amino acid sequence of the calcium-binding sites is also highly conserved and each site binds three calcium ions (Shapiro *et al*., 1995; Nagar *et al*., 1996; Tamura *et al*., 1998; Pertz *et al*., 1999; Boggon *et al*., 2002). The

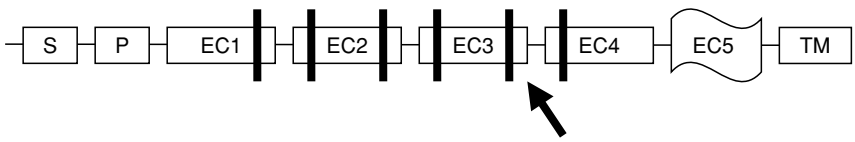



**FIGURE 56.4** Schematic diagram of desmoglein-1, showing the four homologous extracellular domains (EC1–4), a fifth juxtamembrane domain (EC5) that is not as well conserved within or among the cadherins and the putative calcium-binding sites (vertical bars). The exfoliative toxins disrupt cell-to-cell adhesion in the epidermis by hydrolyzing a single peptide bond between glutamic acid-381 and glycine-382 located between EC3 and EC4 of desmoglein-1 (arrow). S, Signal peptide; P, Propeptide; TM, Transmembrane domain.

toxins cleave desmoglein-1 between EC3 and EC4 after glutamic acid residue 381 (counting from the initiating methionine residue of both mouse and human desmoglein-1), precisely in one of the calcium-binding sites of desmoglein-1. This results in a conformation change in desmoglein-1, leading to dysfunction of the molecule and loss of intercellular adhesion within the epidermis (Hanakawa *et al*., 2003; Hanakawa *et al*., 2004).

The reaction between the exfoliative toxins and desmoglein-1 does not require any other factor—incubation of the toxins with recombinant extracellular domain of desmoglein-1 alone results in a dose-dependent degradation of the latter (Amagai *et al*., 2000; Amagai *et al*., 2002). Furthermore, when the serine residue of the active site is replaced with alanine, the toxins are still able to bind but unable to cleave desmoglein-1, supporting previous speculations that the toxins act as serine proteases. On the other hand, broad spectrum serine protease inhibitors such as diiopropylfluorophosphate (DFP), 3,4-dichloroisocoumarin (DCI), and α2-macroglobulin do not efficiently inhibit the toxins, suggesting that the catalytic site is either inaccessible or inactive. Current data suggest that the toxin must first bind to a very specific region on desmoglein-1 like a key in a lock, which in turn results in a conformation change (the proposed 180° flip of the proline-glycine bond in ETA and its equivalent valine-glycine bond in ETB) in the toxin that opens and/or correctly aligns the active site of the toxin with the cleavage site on desmoglein-1.

Using truncated mutants of desmoglein-1, Hanakawa and colleagues (2004) showed that the second and third extracellular domains of desmoglein-1, particularly sequences between amino acids 214–398, are essential for cleavage by the exfoliative toxins. Insertion of these sequences into the homologous human desmoglein-3, which the toxins are unable to hydrolyze, results in cleavage. The authors further showed that five amino acids on a loop in the third extracellular domain around 110 residues upstream from the cleavage site are essential for binding the toxin (Hanakawa *et al*., 2004). Substituting these five amino acids from human desmoglein-1 into canine desmoglein-1, which is not susceptible to the exfoliative toxins, resulted in hydrolysis of the latter (Figure 56.5).

However, unlike typical serine proteases, such as trypsin and chymotrpysin, the exfoliative are not only sequence-specific, but are also dependent on the three-dimensional structure of their substrate (Hanakawa *et al*., 2003). The toxins are unable to hydrolyze desmoglein-1 that has been heat-denatured at 56°C or 80°C for 1 hour or preincubated at low or high pH. Similarly, depletion of calcium from desmoglein-1 by

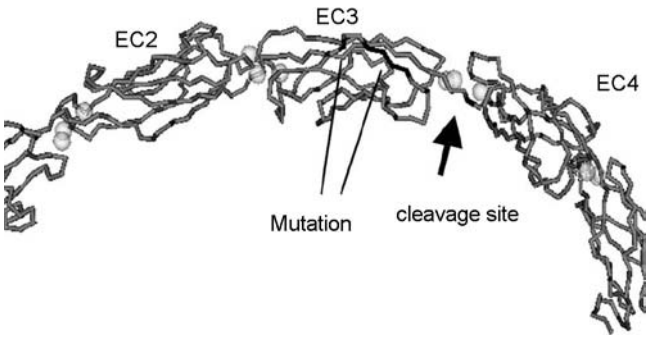



**FIGURE 56.5** Structure of canine desmoglein-1 based on the c-cadherin crystal structure. Canine desmoglein-1 is not susceptible to the exfoliative toxins. However, mutation of 5 critical amino acids between residues 271–277 on a loop proximal to the cleavage site in the third extracellular domain (EC3) to human sequences allows cleavage (thick arrow) of canine desmoglein-1 by the exfoliative toxins. (Reproduced from Hanakawa *et al.* (2004) and by permission of *Journal of Biological Chemistry*, American Society for Biochemistry and Molecular Biology)

preincubating with ethylenediamine tetra-acetic acid (EDTA) causes an irreversible conformational change in desmoglein-1, as demonstrated by antibodies that recognize specific conformational epitopes on desmoglein-1, immunofluorescence, circular dichronism, and tryptophan fluorometry (Hanakawa *et al*., 2003). The exfoliative toxins are unable to hydrolyze calcium-depleted desmoglein-1, even after calcium is added back into the assay. Together, these results suggest that the exfoliative toxins bind to a very specific region on desmoglein-1 that is dependent on both the primary amino acid sequence and the conformation of desmoglein-1, like a key in a lock. This results in a conformational change in the toxins (the proposed 180° flip in the proline-192-glycine-193 bond in ETA or its equivalent valine-183-glycine-184 in ETB) that will expose and/or align its active site with the specific cleavage site on desmoglein-1. This highly specific interaction explains both how the toxins are able to target one specific molecule within the body and why only certain species are affected.

## The toxins and the immune system

Whether staphylococcal exfoliative toxins possess superantigenic activity is currently under considerable debate. Superantigens are a group of bacterial and viral proteins that are able to bind directly to the Major Histocompatibility Complex (MHC) on the surface of antigen presenting cells outside the antigen binding groove (Dellabona *et al*., 1990) and cross-link with the variable Vβ region of the β-chain of the T cell receptor (Fields *et al*., 1996; Kappler *et al*., 1989), to potentially activate up to 20% of all T cells (Skov and Baadsgaard,

1995). *S. aureus* produces several superantigens, including toxic shock syndrome toxin-1 (Prasad *et al*., 1993; Acharya *et al*., 1994) and the staphylococcal enterotoxins (Baker and Acharya, 2004; Alouf and Müller-Alouf, 2003), and new superantigens continue to be discovered (Letertre *et al*., 2003; Omoe *et al*., 2004).

Initial reports indicated that the exfoliative toxins had mitogenic activity toward murine T lymphocytes (Morlock *et al*., 1980), and ETA was shown to preferentially stimulate Vβ2 human (Choi *et al*., 1990) and Vβ3 murine T cells (Herman *et al*., 1990). Furthermore, the toxins were able to activate murine macrophages to release high levels of tumor necrosis factor-α, interleukin-6, and nitric oxide to cause contact-dependent cytotoxicity in transformed embryo fibroblast cells, although the level of killing is lower than that for staphylococcal toxic shock syndrome toxin-1 and the enterotoxins (Fleming *et al*., 1991). However, others using recombinant ETA cloned into either a non-toxin producing strain of *S. aureus* (Fleischer and Bailey, 1992) or *Escherichia coli* (Plano *et al*., 2000) were unable to demonstrate any mitogenic activity towards human or murine T lymphocytes. The authors proposed that the superantigenic activity observed in previous studies was probably due to contamination by other staphylococcal enterotoxins at levels too low to be detectable by conventional biochemical methods (Fleischer and Bailey, 1992; Fleischer *et al*., 1995), as has been previously demonstrated with staphylococcal protein A (Schrezenmeier and Fleischer, 1987).

The authors further supported their argument with the following observations: (i) the characteristic symptoms of cytokines (including rash, fever, and hypotension) induced by the action on lymphocytes of other superantigens, such as toxic shock syndrome toxin-1 and the enterotoxins, is not seen with the exfoliative toxins; (ii) histological examination of skin from patients with SSSS does not show any recruitment of immune system cells; (iii) although the exfoliative toxins are similar to the other superantigens in that they are globular proteins and have similar molecular masses of 24 to 30 kDa, the degree of primary sequence and structural homology such as that seen between other superantigens is poor (Marrack and Kappler, 1990); (iv) none of the other known superantigens possess the protease activity of the exfoliative toxins; (v) ETA and ETB purified from *S. aureus* strains could stimulate spleen cells of transgenic mutant mice that were deficient in the expression of MHC class II antigens (Chapes *et al*., 1993), as well as MHC class II-deficient macrophages (Beharka *et al*., 1994); and (vi) it has not been possible to precipitate MHC class II molecules with commercial ETA, possibly because the toxins do not bind to the molecule.

On the other hand, there is some evidence that the exfoliative toxins possess a unique and specific superantigenic activity, which is separate from their enzymatic activity. Vath and colleagues (1997) cloned ETA into a superantigen-free strain of *S. aureus* and showed that the purified toxin had mitogenic activity toward T cells. The authors argued that since superantigenic activity could be demonstrated only after cloning ETA into the superantigen-free strain of *S. aureus*, ETA must be the source of the superantigenic activity and contamination by other superantigens was, therefore, unlikely. Also, recombinant ETA with a mutated active site, Ser195Cys-ETA, possessed a similar mitogenic activity but no exfoliative action when injected into newborn mice, indicating that the mitogenic activity was separate from the exfoliative activity. This dual action is also seen with the separate emetic activity and mitogenicity of the enterotoxins (Bohach *et al*., 1990) and with the lethality and mitogenicity of toxic shock syndrome toxin-1 (Murray *et al*., 1996). In contrast to other known superantigens, the superantigenic activity of the exfoliative toxins is considered to be unique because they induce selective polyclonal expansion of Vβs 3, 12, 13.2, 14, 15, and 17 (but not Vβ2), and only those murine Vβ T cells that are highly homologous to human forms are induced (Monday *et al*., 1999).

The exfoliative toxins are also able to stimulate the skin-associated lymphoid tissue (SALT), a part of the immune system that deals with antigenic challenges common to the skin and includes antigen-presenting Langerhans' cells, keratinocytes, and T cell subsets with skin-homing receptors (Streilein, 1989). *In vitro* studies have shown that the toxins can activate murine splenic T cells in the presence of Langerhans' cells and MHC class II-bearing keratinocytes to produce a range of cytokines, including IL-1 and tumor necrosis factor-α, capable of stimulating other accessory and immune system cells (Tokura *et al*., 1994). Furthermore, binding of the exfoliative toxins to Langerhans' cells results in dose-dependent depletion of these cells in cultured mouse skin, presumably due to migration of the cells to draining lymph nodes, where they may act as antigen-presenting cells for T lymphocytes (Pickard *et al*., 1994). The release of cytokines following activation of SALT may be responsible for the erythematous rash seen in SSSS, as has been proposed for the toxic shock syndrome toxin-1 in toxic shock syndrome (Leung *et al*., 1995a) and with the superantigens of group A streptococci in the development of the characteristic cutaneous swelling, erythema, and desquamation seen in streptococcal infections (Stevens *et al*., 1989). However, the lack of immune cells on histological specimens from patients

with SSSS cannot be easily explained and requires more research.

When compared to ETA, ETB is considered to be more pyrogenic and enhances susceptibility to lethal shock in rabbits (Monday *et al*., 1999). ETB is also more frequently isolated in generalized compared to localized SSSS and can cause generalized SSSS in apparently healthy adults (Opal *et al*., 1988). Furthermore, flow cytometry analysis showed that T cells stimulated by ETB show a corresponding population of T cell receptors on their surface bearing the appropriate Vβ (Monday *et al*., 1999). On the other hand, ETA appears to decrease the number of expected Vβ receptors on the surface of T cells, suggesting that ETA may down-regulate T cell receptors on the expanded set of T cells, a process also thought to occur with other superantigens (White *et al*., 1989). Despite these differences, both ETA and ETB produce identical skin lesions in humans and mice. There have been no studies on the superantigenic activity of other *S. aureus* or *S. hyicus* exfoliative toxins.

## FUTURE DIRECTIONS

Discovery of the mechanism of action of the exfoliative toxins and identification of their specific epidermal target has opened a whole new area for both academic and clinical research. Identification of a specific substrate means that a simple laboratory assay is now available to study different chemical and biological properties of the toxins without the need for live animal models. Such an assay could, for example, be used to determine how ETC from *S. aureus* and *S. hyicus* exfoliative toxin A (SHETA), which do not do not possess the serine-histidine-aspartate active site, are still able to induce exfoliation in their respective animal models.

Identification of desmoglein-1 as the substrate for the toxins should lead to the development of rapid, sensitive, and specific diagnostic assays that can be performed routinely in hospital laboratories and can detect and quantify the toxins in serum or other biological fluids from patients suspected with SSSS within a few hours. It should also be possible to develop new targeted anti-toxin therapies using analogues of desmoglein-1 that bind and inactivate the toxins before they reach the epidermis, as has been shown with *Listeria monocytogenes*, whose ability to invade epithelial cells can be inhibited by N-terminal fragments and recombinant peptides of E-cadherin (Lecuit *et al*., 1999). While such therapies may not be suitable for most uncomplicated cases of generalized SSSS, they may be lifesaving in high-risk patients, such as premature neonates and adults with serious underlying illnesses, and in cases due to multiresistant *S. aureus* (Yokota *et al*., 1996; Ackland *et al*., 1999), where antibiotic treatment alone may not be sufficient.

The exfoliative toxins may also play an important role in a wide range of other diseases where superantigens have been implicated. The ability of the exfoliative toxins to activate the SALT component of the immune system, for example, may have important consequences on various dermatological conditions, including cutaneous T cell lymphoma and atopic dermatitis. ETA is capable of inducing the proliferation of Vβ2.1-bearing cutaneous T cell lymphoma cells *in vitro*, and this proliferative response is enhanced by adding IL-1. This fits in with observations showing that activation of SALT by ETA results in IL-1 release (Tokura *et al*., 1994), and IL-1, along with IL-6, plays an important role in enhancing the activity of other superantigens (Kotb *et al*., 1990). Similarly, more than 90% of patients with atopic dermatitis are colonized with *S. aureus*, and their role in the pathogenesis of atopic dermatitis is supported by studies showing that eradication of *S. aureus* with antistaphylococcal antibiotics results in clinical improvement of the skin condition (Lever *et al*., 1988; Abeck and Mempel, 1998; Taskapan and Kumar, 2000; Zollner *et al*., 2000). Most *S. aureus* isolates from patients with atopic dermatitis release superantigens (Leung *et al*., 1993; McFadden *et al*., 1993; Zollner *et al*., 2000), and it has been speculated that superantigens bind to surface MHC class II molecules found on keratinocytes during inflammation and activate polyclonal T cells locally (Strange *et al*., 1994). Other conditions where staphylococcal superantigens may play an important role in pathogenesis include Kawasaki disease, a multisystem disorder in children that is characterized by fever, rash, conjunctivitis, mucosal inflammation, erythematous peeling of the hands and feet, and, rarely, coronary artery aneurysms (Melish, 1992; Leung *et al*., 1995b), hematogenously acquired staphylococcal nephritis (Koyama *et al*., 1995; Verba and Tarkowski, 1996), staphylococcal septic arthritis (Bremmel and Tarkowski, 1995; Zhao *et al*., 1996), rheumatoid arthritis (Paliard *et al*., 1991), multiple sclerosis (Brocke *et al*., 1994; Stinnisen *et al*., 1995), contact sensitivity (Saloga *et al*., 1995), nasal polyposis (Bachert *et al*., 2003; Bernstein *et al*., 2003), guttate and chronic plaque psoriasis (Lewis *et al*., 1993; Yokote *et al*., 1995), and sudden infant death syndrome (Blackwell *et al*., 1993; Lee *et al*., 1987; Morris *et al*., 1987). The role of the exfoliative toxins in these conditions, if any, needs to be established.

Understanding the action of the exfoliative toxins also has wider applications. For example, the toxins could be used in a controlled manner to induce localized exfoliation of offending superficial skin lesions

with minimal scarring. In addition, recent unpublished data show that the toxins preferentially concentrate in the skin of both susceptible (Balb/c mice) and non-susceptible (rats) newborn species, suggesting that the targeting and binding region of the toxins is different to the proteolytic region (Plano, 2004). Therefore, toxin analogues that bind but do not cleave desmoglein-1 could be used to stabilize the protein and protect it against, for example, destruction by autoantibodies in pemphigus foliaceus. Similarly, the targeting domain of the toxins could be used to deliver drugs, such as chemotherapy, to a localized region within the epidermis, with minimal systemic side effects.

Another area that requires further research involves identification of factors that lead to development of the disease. At least one-third of the general population carries *S. aureus* commensally and around 5% of these strains produce exfoliative toxin, but only a very small proportion of carriers develop SSSS. Polymorphisms in the desmoglein-1 gene may be one possible explanation. It is also possible that a triggering factor in either the organism or the host may be required for toxin production, or that the organism is constantly producing the toxins but protective mechanisms in the host, either locally or in the blood, prevent the toxins from reaching the epidermis. Of particular interest is the mechanism by which the toxins are able to cross the protective mucous membranes and endothelium to enter the bloodstream. A recent study using staphylococcal toxic shock syndrome toxin-1 and enterotoxins A and B has identified a highly conserved region that is essential for crossing the gut lining, and a synthetic peptide based on the conserved region (KKKV-TAQELD) was able to significantly reduce transcytosis (Shupp *et al*., 2002). The exfoliative toxins may also possess such a conserved region, which may provide a target for future vaccine development to prevent SSSS.

## CONCLUSION

Staphylococcal scalded skin syndrome is a relatively uncommon but potentially fatal blistering skin disorder, particularly in adults who have serious underlying disease and in young infants who are prone to secondary complications of exfoliation. The exfoliative toxins of *Staphylococcus aureus* are responsible for the condition and the mechanism by which they produce exfoliation is unique and highly specific. The toxins act as atypical serine proteases, which are inactive in their native state and require activation at their site of action. Binding to desmoglein-1, a transmembrane glycoprotein of desmosomes in the epidermis, produces a conformation change in the toxins, which exposes their active site and allows cleavage of desmoglien-1 at a critical site, resulting in exfoliation of the superficial epidermis and disruption of the skin barrier. There is also evidence that the exfoliative toxins possess a unique and specific superantigenic activity, which is separate from their enzymatic activity. Understanding the mechanism of action of the exfoliative toxins and identification of their epidermal target has not only helped us understand the pathogenesis of SSSS, but should also provide useful information on normal skin physiology and other toxin-mediated diseases. In the near future, the toxins will have other useful benefits in dermatology and therapeutics. However, many questions remain unanswered, and a lot more work needs to be done before we can close the chapter on the staphylococcal scalded skin syndrome.

## REFERENCES


Abeck, D. and Mempel, M. (1998). *Staphylococcus aureus* colonization in atopic dermatitis and its therapeutic implications. *Br. J. Dermatol*. **139** (Suppl. 53) 13–16.

Acharya, K.R., Passalacqua, E.F., Jones, E.Y., Harlos, K., Stuart, D.I., Brehm, R.D. and H. S. Tranter. (1994). Structural basis of superantigen action inferred from crystal structure of toxic shock syndrome toxin-1. *Nature* **367,** 94–97.

Ackland, K.M., Darvay, A., Griffin, C., Aali, S.A. and Russell-Jones, R. (1999). Staphylococcal scalded skin syndrome in an adult associated with methicillin-resistant *Staphylococcus aureus*. *Br. J. Dermatol*. **14,** 518–520.

Adesiyun, A.A., Lenz, W. and Schaal, K.P. (1991). Exfoliative toxin production by *Staphylococcus aureus* strains isolated from animals and human beings in Nigeria. *Microbiologica* **14,** 357–362.

Ahrens, P. and Andresen, L.O. (2004). Cloning and sequence analysis of genes encoding *Staphylococcus hyicus* exfoliative toxin types A, B, C, and D. *J. Bacteriol*. **186,** 1833–1837.

Almquist, E. (1891). Pemphigus neonatorum, backteriologisch, und epidemiologisch beleuchtet. *Z. Hyg. Infectionskr*. **10,** 253.

Alouf, J.E. and Müller-Alouf, H. (2003). Staphylococcal and streptococcal superantigens: molecular, biological, and clinical aspects. *Int. J. Med. Microbiol*. **292,** 429–440.

Amagai, M., Matsuyosih, N., Wang, Z.H., Andl, C. and Stanley, J.R. (2000). Toxin in bullous impetigo and staphylococcal scalded skin syndrome targets desmoglein-1. *Nat. Med*. **6,** 1275–1277.

Amagai, M., Yamaguchi, T., Hanakawa, Y., Nishifuji, K., Sugai, M. and Stanley, J.R. (2002). Staphylococcal exfoliative toxin B specifically cleaves desmoglein 1. *J. Investig. Dermatol*. **118,** 845–850.

Anderson, L.O. (1998). Differentiation and distribution of three types of exfoliative toxin produced by *Staphylococcus hyicus* from pigs with exudative epidermitis. *FEMS Immunol. Med. Microbiol*. **20,** 301–310.

Angst, B.D., Marcozzi, C. and Magee, A.I. (2001). The cadherin superfamily. Diversity in form and function. *J. Cell Sci*. **114,** 629–641.

Annunziato, D. and Goldblum, L.M. (1978). Staphylococcal scalded skin syndrome. *Am. J. Dis. Child*. **132,** 1187–1188.

Arbuthnott, J. P. and Billcliffe, B. (1976). Qualitative and quantitative methods for detecting staphylococcal epidermolytic toxin. *J. Med. Microbiol*. **9,** 191–201.

Arbuthnott, J. P., Billcliffe, B. and Thompson, W.D. (1974). Isoelectric focusing studies of staphylococcal epidermolytic toxin. *FEBS Lett*. **46,** 92–95.

Arbuthnott, J.P. (1981). Characterization of the epidermolytic toxins of *Staphylococcus aureus*. In: *The Staphylococci—Proceedings of the Alexander Ogsten Centennial Conference* (eds. A. MacDonald and G. Smith), pp. 109–118. Aberdeen University Press, Aberdeen, United Kingdom.

Arbuthnott, J.P., Gemmell, C.G., Kent, J. and Lyell, A. (1969). Hemolysin and enzyme patterns of coagulase-positive staphylococci isolated from toxic epidermal necrolysis, Ritter's disease, and impetigo contagiosa. *J. Med. Microbiol*. **2,** 479–487.

Arbuthnott, J.P., Kent, J. and Noble, W.C. (1973). The response of hairless mice to staphylococcal epidermolytic toxin. *Br. J. Dermatol*. **88,** 481–485.

Arbuthnott, J.P., Kent, J., Lyell, A. and Gemmell, C.G. (1971). Toxic epidermal necrolysis produced by an extracellular product of *Staphylococcus aureus. Br. J. Dermatol*. **85,** 145–149.

Archer, G.L. (1998). *Staphylococcus aureus*: a well-armed pathogen. *Clin. Infect. Dis*. **26,** 1179–1181.

Arkwright, P.D., Daniel, T.O., Sanyal, D., David, T.J. and Patel, L. (2002). Age-related prevalence and antibiotic resistance of pathogenic staphylococci and streptococci in children with infected atopic dermatitis at a single-specialty center. *Arch. Dermatol*. **138,** 939–941.

Ayliffe, G.A. (1997). The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus. Clin. Infect. Dis*. **24** (Suppl. 1) S 74–79.

Bachert, C., van Zele, T., Gevaert, P., De Schrijver, L. and Van Cauwenberge, P. (2003). Superantigens and nasal polyps. *Curr. Allergy Asthma Rep*. **3,** 523–531.

Bailey, C.J. and Redpath, M.B. (1992). The esterolytic activity of epidermolytic toxins. *Biochem. J.* **284,** 177–180.

Bailey, C.J. and Smith, T.P. (1990). The reactive serine residue of epidermolytic toxin A. *Biochem. J.* **269,** 535–537.

Bailey, C.J., De Azavedo, J. and Arbuthnott, J.P. (1980). A comparative study of two serotypes of epidermolytic toxins from *Staphylococcus aureus. Biochim. Biophys. Acta* **624,** 111–120.

Bailey, C.J., Lockhart, B.P., Redpath, M.B. and Smith, T.P. (1995). The epidermolytic (exfoliative) toxins of *Staphylococcus aureus. Med. Microbiol. Immunol*. **184,** 53–61.

Baker, M.D. and Acharya, K.R. (2004). Superantigens: structure-function relationships. *Int. J. Med. Microbiol*. **293,** 529–537.

Barbosa, J.A.R.G., Saldanha, J.W. and Garratt, R.C. (1996). Novel features of serine protease active sites and specificity pockets: sequence analysis and modeling studies of glutamate-specific endopeptidases and epidermolytic toxins. *Protein Eng*. **9,** 591–601.

Becker, K., Haverkamper, G., von Eiff, C., Roth, R. and Peters, G. (2001). Survey of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin-1 gene in non-*Staphylococcus aureus* species. *Eur. J. Clin. Microbiol. Infect. Dis*. **20,** 407–409.

Beharka, A.A., Armstrong, J.W., Iandolo, J.J and Chapes, S.K. (1994). Binding and activation of major histocompatibility complex class II-deficient macrophages by staphylococcal exotoxins. *Infect. Immun*. **62,** 3907–3915.

Bernstein, J.M., Ballow, M., Schlievert, P.M., Rich, G., Allen, C. and Dryja, D. (2003). A superantigen hypothesis for the pathogenesis of chronic hyperplastic sinusitis with massive nasal polyposis. *Am. J. Rhinol*. **17,** 321–326.

Blackwell, C.C., Saadi, A.T., Raza, M.W., Wier, D.M. and Busuttil, A. (1993). The potential role of bacterial toxins in sudden infant death syndrome. *Int. J. Leg. Med*. **105,** 333–338.

Boggon, T.J., Murray, J., Chappuis-Flament, S., Wong, E., Gumbiner, B.M. and Shapiro, L. (2002) C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* **296,** 1308–1313.

Bohach, G.A., Fast, D.J., Nelson, R.D. and Schlievert, P.M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxin shock syndromes and related diseases. *Crit. Rev. Microbiol*. **17,** 251–272.

Bremmell, T. and Tarkowski, A. (1995). Preferential induction of septic arthritis and mortality by superantigen-producing staphylococci. *Infect. Immun*. **63,** 4185–4187.

Brocke, S., Veromaa, T., Weissman, I.L., Gijbels, K. and Steinman, L. (1994). Infection and multiple sclerosis: a possible role for superantigens? *Trends Microbiol*. **2,** 250–254.

Cavarelli, J., Prevost, G., Bourguet, W., Moulinier, L., Chevrier, B., Delagoutte, B., Bilwes, A., Mourey, L., Rifai, S., Piemont, Y. and Moras, D. (1997). The structure of *Staphylococcus aureus* epidermolytic toxin A, an atypic serine protease, at 1.7Å resolution. *Structure* **5,** 813–824.

Chapes, S., Hoynowski, S.M., Woods, K.M., Armstrong, J.W., Beharka, A.A. and Iandolo, J. (1994). Staphylococcus-mediated T cell activation and spontaneous natural killer cell activity in the absence of major histocompatibility complex class II molecules. *Infect. Immun*. **61,** 4013–4016.

Chiller, K., Selkin, B.A. and Murakawa, G.J. (2001). Skin microflora and bacterial infections of the skin. *J. Investig. Dermatol. Symp. Proc*. **6,** 170–174.

Choi, Y., Kotzin, B., Herron, L., Callahan, J., Marrack, P. and Kappler, J. (1990). Interaction of *Staphylococcus aureus* toxin superantigens with human T cells. *Proc. Natl. Acad. Sci. USA* **86,** 8941–8945.

Cribier, B., Piemont, Y. and Grosshans, E. (1984). Staphylococcal scalded skin syndrome in adults: a clinical review illustrated with a case. *J. Am. Acad. Dermatol*. **30,** 319–324.

Dajani, A.S. (1972). The scalded skin syndrome: relation to phage group II staphylococci. *J. Infect. Dis*. **125,** 548.

Dancer, S.J. and Noble, W.C. (1991). Nasal, axillary, and perineal carriage of *Staphylococcus aureus* among women: identification of strains producing epidermolytic toxin. *J. Clin. Pathol*. **44,** 681–684.

Dancer, S.J., Garratt, R., Saldanha, J., Jhoti, H. and Evans, R. (1990). The epidermolytic toxins are serine proteases. *FEBS Lett*. **268,** 129–132.

Dancer, S.J., Simmons, N.A., Poston, S.M. and Noble, W.C. (1988). Outbreak of staphylococcal scalded skin syndrome among neonates. *J. Infect*. **16,** 87–103.

Darmstadt, G.L. (1997). Oral antibiotic therapy for uncomplicated bacterial skin infections in children. *Pediatr. Infect. Dis. J*. **16,** 227–240.

De Azavedo, A. and Arbuthnott, J.P. (1981). Prevalence of epidermolytic toxin in clinical isolates of *Staphylococcus aureus. J. Med. Microbiol*. **14,** 341–344.

Dedobbeleer, G. and Achten, G. (1975). Staphylococcal scalded skin syndrome. An ultrastructural study. *J. Cutaneous Pathol*. **2,** 91–96.

Dellabona, P., Peccoud, J., Kappler, J., Marrack, P., Benoist, C. and Mathis, D. (1990). Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* **62,** 1115–1121.

Doern, G.V., Jones, R.N., Pfaller, M.A., Kugler, K.C. and Beach, M.L. (1999). Bacterial pathogens isolated from patients with skin and soft tissue infections: frequency of occurrence and antimicrobial susceptibility patterns from the SENTRY Antimicrobial Surveillance Program (United States and Canada, 1997). SENTRY Study Group (North America). *Diagn. Microbiol. Infect. Dis*. **34,** 65–72.

Drapeau, G.R. (1978). The primary structure of *Staphylococcus aureus. Can. J. Biochem*. **56,** 534–544.

Drews, M.B., Ludwig, A.C., Leititis, J.U. and Daschner, FD. (1995). Low birth weight and nosocomial infection of neonates in a neonatal intensive care unit. *J. Hosp. Infect*. **30,** 65–72.

Elias, P.M., Fritsch, P., Dahl, M.V. and Wolff, K. (1975). Staphylococcal toxic epidermal necrolysis: pathogenesis and studies on the sub-

cellular site of action of exfoliatin. *J. Investig. Dermatol*. **65,** 501–512.

Elsner, P. and Hartman, A.A. (1988). Epidemiology of ETA- and ETB-producing staphylococci in dermatological patients. *Zentbl. Bacteriol. Mikrobiol. Hyg. Ser. A* **268,** 534.

Failla, D.M. and Pankey, G.A. (1994). Optimum outpatient therapy of skin and skin structure infections. *Drugs* **48,** 172–178.

Ferscht, A.R. (1985). *Enzyme Structure and Mechanism*, 2nd ed. W.H. Freeman & Co., New York.

Fields, B.A., Malchiodi, E.L., Li, H., Ysern, X., Stauffacher, C.V. and Schlievert, P.M. (1996). Crystal structure of a T cell receptor beta-chain complexed with a superantigen. *Nature* **384,** 188–192.

Finch, R. (1988). Skin and soft-tissue infections. *Lancet* **i,** 164–168.

Fleischer, B. and Bailey, C.J. (1992). Recombinant epidermolytic (exfoliative) toxin A of *Staphylococcus aureus* is not a superantigen. *Med. Microbiol. Immunol*. **180,** 273–278.

Fleischer, B., Gerlach, D., Fuhrmann, A. and Schmidt, K.H. (1995). Superantigens and pseudosuperantigens of Gram-positive cocci. *Med. Microbiol. Immunol*. **184,** 1–8.

Fleming, S.D., Iandolo, J.J. and Chapes, S.K. (1991). Murine macrophage activation by staphylococcal exotoxins. *Infect. Immun*. **59,** 4049–4055.

Fleurette, J. and Ritter, J. (1980). Prevalence des souches de *Staphylococcus aureus* productrices d'exfoliatine dans le groupe bacteriopagique II. *Ann. Microbiol*. **1131B,** 175–183.

Fritsch, P., Elias, P. and Varga, J. (1976). The fate of staphylococcal exfoliatin in newborn and adult mice. *Br. J. Dermatol*. **95,** 275–284.

Gales, A.C., Jones, R.N., Pfaller, M.A., Gordon, K.A. and Sader, H.S. (2000). Two-year assessment of the pathogen frequency and antimicrobial resistance patterns among organisms isolated from skin and soft tissue infections in Latin American hospitals: results from the SENTRY antimicrobial surveillance program, 1997–98. *SENTRY Study Group. Int. J. Infect. Dis*. **4,** 75–84.

Ginsburg, C.M. (1991). Staphylococcal toxin syndromes. *Pediatr. Infect. Dis. J*. **10,** 319–321.

Gravet, A., Colin, D.A., Keller, D., Girardot, R., Monteil, H. and Prevost, G. (1998) Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. *FEBS Lett*. **436,** 202–208.

Gravet, A., Couppie, P., Meunier, O., Clyti, E., Moreau, B., Pradinaud, R., Monteil, H. and Prevost, G. (2001). *Staphylococcus aureus* isolated in cases of impetigo produces both epidermolysin A or B and LukE-LukD in 78% of 131 retrospective and prospective cases. *J. Clin. Microbiol*. **39,** 4349–4356.

Griego, R.D., Rosen, T., Orengo, I.F. and Wolf, J.E. (1995). Dog, cat, and human bites: a review. *J. Am. Acad. Dermatol*. **33,** 1019–1029.

Grohskopf, L.A., Sinkowitz-Cochran, R.L., Garrett, D.O., Sohn, A.H., Levine, G.L., Siegel, J.D., Stover, B.H., Jarvis, W.R. and the Pediatric Prevention Network. (2002). A national point-prevalence survey of pediatric intensive care unit acquired infections in the United States. *J. Pediatr*. **140,** 432–438.

Hacker, J., Blum-Oehler, G., Hochhut, B.and Dobrindt, U. (2003). The molecular basis of infectious diseases: pathogenicity islands and other mobile genetic elements. *Acta Microbiol Immunol Hung*. **50,** 321–330.

Hanakawa, Y., Schechter, N.M., Lin, C., Garza, L., Li, H., Yamaguchi, T., Fudaba, Y., Nishifuji, K., Sugai, M., Amagai, M. and Stanley, J.R. (2002). Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome. *J. Clin. Invest*. **110,** 53–60.

Hanakawa, Y., Schechter, N.M., Lin, C., Nishifuji, K., Amagai, M. and Stanley, J.R. (2004). Enzymatic and molecular characteristics of the efficiency and specificity of exfoliative toxin cleavage of desmoglein 1. *J. Biol. Chem*. **279,** 5268–5277.

Hanakawa, Y., Selwood, T., Woo, D., Lin, C., Schechter, N.M. and Stanley, J.R. (2003). Calcium-dependent conformation of desmoglein 1 is required for its cleavage by exfoliative toxin. *J. Invest. Dermatol*. **121,** 383–389.

Herman, A., G. Croteau, R. P. Sekaley, J. Kappler, and P. Marrack. 1990. HLA-DR alleles differ in their ability to present staphylococcal enterotoxins to T cells. *J. Exp. Med*. **172,** 709–717.

Hoffmann, R., Lohner, M., Bohm, N., Schaefer, H.E. and Letitis, J. (1994). Staphylococcal scalded skin syndrome and consecutive septicemia in a preterm infant. *Pathol. Res. Pract*. **190,** 77–81.

Huang, S.S. and Platt, R. (2003). Risk of methicillin-resistant *Staphylococcus aureus* infection after previous infection or colonization. *Clin. Infect. Dis*. **36,** 281–285.

Hudome, S.M. and Fisher, M.C. (2001). Nosocomial infections in the neonatal intensive care unit. *Curr. Opin. Infect. Dis*. **14,** 303–307.

Hussain, F.M., Boyle-Vavra, S. and Daum, R.S. (2001). Community-acquired methicillin-resistant *Staphylococcus aureus* colonization in healthy children attending an outpatient pediatric clinic. *Pediatr. Infect. Dis. J*. **20,** 763–767.

Itani, O., Crump, R., Minoumi, F. and Tunnessen, W.W. (1992). Picture of the month: staphylococcal scalded skin syndrome. *Am. J. Dis. Child*. **146,** 424–425.

Jackson, M.P. and Iandolo, J.J. (1986). Cloning and expression of exfoliative toxin B gene from *Staphylococcus aureus. J. Bacteriol*. **166,** 574–580.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J. and Vandenesch, F. (2003). Relationships between *Staphylococcus aureus* genetic background, Virulence Factors, *agr* Groups (Alleles), and human disease. *Infect. Immun*. **70,** 631–641.

Jaulhac, B., Piemont, Y. and Prevost, G. 1994. Staphylococcal exfoliative toxins induce proteolytic activity when combined with epidermis. In: *Staphylococci and Staphylococcal Infections*. *Proceedings of the 7th International Symposium* (eds. R. Mollby, J.I. Flock, C.E. Nord and B. Christensson). pp. 280-282. Gustav Fischer Verlag, Stuttgart.

Ji, G., Beavis, R. and Novick, R.P. (1997). Bacterial interference caused by autoinducing peptide variants. *Science* **276,** 2027–2030.

Ji, G., Beavis, R.C. and Novick, R.P. (1995). Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl. Acad. Sci. USA*. **92,** 12055–12059.

Jones, M.E., Schmitz, F.J., Fluit, A.C., Acar, J., Gupta, R. and Verhoef, J. (1999). Frequency of occurrence and antimicrobial susceptibility of bacterial pathogens associated with skin and soft tissue infections during 1997 from an International Surveillance Programme. SENTRY Participants Group. *Eur. J. Clin. Microbiol. Infect. Dis*. **18,** 403–408.

Kallman, J., Kihlstrom, E., Sjoberg, L. and Schollin, J. (1997). Increase of staphylococci in neonatal septicaemia: a fourteen-year study. *Acta Paediatr*. **86,** 533–538.

Kaplan, M.H., Chmel, H., Hseih, H., Staphens, A. and Brinsko, V. (1986). Importance of exfoliative toxin A producing *Staphylococcus aureus* strains isolated from clustered epidemics of neonatal pustulosis. *J. Clin. Microbiol*. **23,** 83–91.

Kappler, J., B. Kotzin, L. Herron, E. W. Gelfand, R. D. Bigler, A. Boylston, S. Carrel, D. N. Posnett, and Choi, Y. (1989). V beta-specific stimulation of human T cells by staphylococcal toxins. *Science* **244,** 811–813.

Kapral, F.A. (1975). Staphylococcus exfoliatin. In: *Microbiology* (ed. D. Schlessinger), pp. 263–266. American Society for Microbiology, Washington, D.C.

Kapral, F.A. and Miller, M.M. (1971). Product of *Staphylococcus aureus* responsible for the scalded skin syndrome. *Infect. Immun*. **4,** 541–545.

Kluytmans, J.A.J.W., Mouton, J.W., Ijzerman, E.P.F., Vandenbroucke-Grauls, C.M., Maat, A.W., Wagenvoort, J.H. and Verbrugh, H.A. (1995). Nasal carriage of *Staphylococcus aureus* as a major risk factor for wound infections after cardiac surgery. *J. Infect. Dis*. **171,** 216–219.

Kondo, I. (1976). Staphylococcal exfoliatin A and B. In: *Saikin Dokuso Kenkyu* (ed. I. Kato), pp. 82–95. Kyouritsu Press, Tokyo.

Kondo, I., Sakurai, S. and Sarai, Y. (1974). New type of exfoliatin obtained from staphylococcal strains belonging to phage groups other than group II, isolated from patients with impetigo and Ritter's disease. *Infect. Immun*. **10,** 851–861.

Kondo, I., Sakurai, S. and Sarai, Y. (1975a). Purification of exfoliatin produced by *Staphylococcus aureus* of bacteriophage group 2 and its physico-chemical properties. *Infect. Immun*. **8,** 156–164.

Kondo, I., Sakurai, S. Sarai, Y. and Fukati, S. (1975b). Two serotypes of exfoliatin and their distribution in staphylococcal strains isolated from patients with scalded skin syndrome. *J. Clin. Microbiol*. **1,** 397–400.

Korting, H.C. (1994). Critical commentary to staphylococcal scalded skin syndrome and consecutive septicemia in a preterm infant. *Pathol. Res. Pract*. **190,** 82–83.

Kotb, M., Majumdar, G., Tomai, M. and Beachey, E.H. (1990). Accessory cell-independent stimulation of human T cells by streptococcal M protein superantigen. *J. Immunol*. **145,** 1332–1336.

Koyama, A., Kobayashi, M., Yamaguchi, N., Yamagata, K., Takano, K., Nakajima, M., Irie, F., Goto, M., Iragashi, M. and Litsuka, I. (1995). Glomerulonephritis associated with MRSA infection: a possible role of bacterial superantigen. *Kidney Int*. **47,** 207–216.

Ladhani S. (2003). Understanding the mechanism of action of staphylococcal exfoliative toxins. *FEMS Immunol. Med. Microbiol*. **39,** 181–189.

Ladhani S. and Evans, R.W. (1998). Staphylococcal scalded skin syndrome. *Arch. Dis. Child*. **78,** 85–88.

Ladhani, S. (2001). Recent developments in staphylococcal scalded skin syndrome. *Clin. Microbiol. Infect*. **7,** 301–307.

Ladhani, S. and Garbash, M. (2005). Staphylococcal skin infections. *Pediatr. Drugs* (in press).

Ladhani, S. and Joannou, C.L. (2000). Difficulties in diagnosis and management of the staphylococcal scalded skin syndrome. *Pediatr. Infect. Dis. J*. **19,** 819–821.

Ladhani, S. and Newson, T. (2000). Familial outbreak of staphylococcal scalded skin syndrome. *Pediatr. Infect. Dis. J*. **19,** 578–579.

Ladhani, S., Chapple, D.S., Joannou, C.L. and Evans, R.W. (2002). A novel method for rapid production and purification of the staphylococcal exfoliative toxins. *FEMS Microbiol. Lett*. **212,** 35–39.

Ladhani, S., Joannou, C.L., Lochrie, D.P., Evans, R.W. and Poston, S.M. (1999a) Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded skin syndrome. *Clin. Microbiol. Rev*. **12,** 224–242.

Ladhani, S., Joannou, C.L., Poston, S.M. and Evans, R.W. (1999b). Staphylococcal scalded skin syndrome: exfoliative toxin A induces serine protease activity when combined with A431 cells. *Acta. Paediatr*. **88,** 776–779.

Laupland, K.B., Church, D.L., Mucenski, M., Sutherland, L.R. and Davies, H.D. (2003). Population-based study of the epidemiology of and the risk factors for invasive *Staphylococcus aureus* infections. *J. Infect. Dis*. **187,** 1452–1459.

Laupland, K.B., Gregson, D.B., Zygun, D.A., Doig, C.J., Mortis, G. and Church, D.L. (2004) Severe bloodstream infections: a population-based assessment. *Crit. Car. Med*. **32,** 992–997.

Lecuit, M., Dramsi, S., Gottardi, C., Fedor-Chaiken, M., Gumbiner, B. and Cossart P. (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes. EMBO J*. **18,** 3956–3963.

Lee, C.Y., Schmidt, J.J., Johnson-Winger, A.D., Spero, L. and Iandolo, J.J. (1987). Sequence determination and comparison of exfoliative toxin A and toxin B genes from *Staphylococcus aureus. J. Bacteriol*. **169,** 3904–3909.

Letertre, C., Perelle, S., Dilasser, F. and Fach, P. (2003). Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus. J. Appl. Microbiol*. **95,** 38–43.

Leung, D.Y., Gately, M., Trumble, A., Ferguson-Darnell, B., Schlievert, P.M. and Picker, L.J. (1995a). Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production. *J. Exp. Med*. **181,** 747–753.

Leung, D.Y., Harbeck, R., Bina, P., Reiser, R.F., Yang, E., Norris, D.F., Hanifin, J.M. and Scampson, H.A. (1993). Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis: evidence for a new group of allergens. *J. Clin. Invest*. **92,** 1374–1380.

Leung, D.Y., Meissner, C., Fulton, D. and Schlievert, P.M. (1995b). The potential role of bacterial superantigens in the pathogenesis of Kawasaki syndrome. *J. Clin. Immunol*. **15** (Suppl. 6) S 11–17.

Lever, R., Hadley, K., Downey, D. and Mackie, R. (1988). Staphylococcal colonization in atopic dermatitis and the effect of topical mupirocin therapy. *Br. J. Dermatol*. **119,** 189–198.

Lewis, H.M., Baker, B.S., Bokth, S., Powles, A.V., Garioch, J.J., Valdimarsson, H. and Fry, L. (1993). Restricted T cell receptor Vβ gene usage in the skin of patients with guttate and chronic plaque psoriasis. *Br. J. Dermatol*. **129,** 514–520.

Lillibridge, C.D., Melish, M.E. and Glasgow, L.A. (1972). Site of action of exfoliative toxin in the staphylococcal scalded skin syndrome. *Pediatrics* **50,** 728–738.

Loughead, J.L. (1992). Congenital staphylococcal scalded skin syndrome: report of a case. *Pediatr. Infect. Dis. J*. **10,** 319–321.

Lowney, E.D., Baublis, J.V., Kreye, G.M., Harrell, E.R. and McKenzie, A.R. (1967). The scalded skin syndrome in small children. *Arch. Dermatol*. **95,** 359–369.

Lowy, F.D. (1998). *Staphylococcus aureus* infections. *N. Engl. J. Med*. **339,** 520–533.

Lyell, A. (1956). Toxic epidermal necrolysis: an eruption resembling scalding of the skin. *Br. J. Dermatol*. **68,** 355–361.

Lyell, A. (1967). A review of toxic epidermal necrolysis in Britain. *Br. J. Dermatol*. **79,** 662–671.

Lyell, A. (1979). Toxic epidermal necrolysis (the scalded skin syndrome): a reappraisal. *Br. J. Dermatol*. **100,** 69–86.

Machida, K. (1995). Immunological investigations on pathogenesis of staphylococcal scalded skin syndrome. *J. Clin. Pathol*. **43,** 547–556.

Mahoney, M.G., Wang, Z., Rothenberger, K., Koch, P.J., Amagai, M. and Stanley, J.R. (1999). Explanations for the clinical and microscopic localization of lesions in pemphigus foliaceus and vulgaris. *J. Clin. Invest*. **103,** 461–486.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248,** 705–711.

McCallum, H.M. (1972). Action of staphylococcal epidermolytic toxin on mouse skin in organ culture. *Br. J. Dermatol*. **86** (Suppl. 8), 40–41.

McFadden, J.P., Noble, W.C. and Camp, R.D.R. (1993). Superantigenic exotoxin-secreting potential of staphylococci isolated from atopic eczematous skin. *Br. J. Dermatol*. **128,** 631–632.

Melish, M. E. (1992). Kawasaki syndrome: a 1992 update. *Pediatr. Dermatol*. **9,** 335–337.

Melish, M.E. (1982). Staphylococci, streptococci, and the skin: review of impetigo and staphylococcal scalded skin syndrome. *Semin. Dermatol*. **1,** 101–109.

Melish, M.E. and Glasgow, L.A. (1970). The staphylococcal scalded skin syndrome: development of an experimental model. *N. Engl. J. Med*. **282,** 1114–1119.

Melish, M.E., Chen, F.S., Sprouse, S., Stuckey, M. and Murata, M.S. (1981). Epidermolytic toxin staphylococcal infection: toxin levels and host response. In: *Staphylococci and Staphylococcal Infections* (ed. J. Jeljaszewicz), Gustav Fischer Verlag, Stuttgart. 287-298.

Melish, M.E., Glasgow, L.A. and Turner, M.D. (1972). The staphylococcal scalded skin syndrome: isolation and partial characterization of the exfoliative toxin. *Br. J. Dermatol*. **125,** 129–140.

Melish, M.E., Glasgow, L.A., Turner, M.D. and Lillibridge, C.B. (1974). The staphylococcal epidermolytic toxin: its isolation, characterization, and site of action. *Ann. N. Y. Acad. Sci*. **236,** 317–342.

Mengesha, Y.M. and Bennett, M.L. (2002). Pustular skin disorders: diagnosis and treatment. *Am. J. Clin. Dermatol*. **3,** 389–400.

Miyake, Y., Iwai, T., Sugai, M., Miura, K., Suginaka, H. and Nagasaka, N. (1991). Incidence and characterization of *Staphylococcus aureus* from the tongues of children. *J. Dent. Res*. **70,** 1045–1047.

Monday, S.R., Vath, G.M., Ferens, W.A., Deobald, C., Rago, J.V., Gahr, P.J., Monie, D.D., Iandolo, J.J., Chapes, S.K., Davis, W.C., Ohlendorf, D.H., Schlievert, P.M. and Bohach, G.A. (1999). Unique superantigen activity of staphylococcal exfoliative toxins. *J. Immunol*. **162,** 4550–4559.

Morlock, B.A., Spero, L. and Johnson, A.D. (1980). Mitogenic activity of staphylococcal exfoliative toxin. *Infect. Immun*. **30,** 381–384.

Morris, J.A., Haran, D. and Smith, A. (1987). Hypothesis: common bacterial toxins are a possible cause of sudden infant death syndrome. *Med. Hypotheses* **22,** 211–222.

Murono, K., Fujita, K. and Yoshioka, H. (1988). Microbiological characteristics of exfoliative toxin-producing *Staphylococcus aureus*. *Pediatr. Infect. Dis. J*. **7,** 313–315.

Murray, D.L., Earhart, C.A., Mitchell, D.T., Ohlandorf, D.H., Novick, R.P. and Schlievert, P.M. (1996). Localization of biologically important regions on toxic shock syndrome toxin-1. *Infect. Immun*. **64,** 371–374.

Mylotte, J.M., McDermott, C. and Spooner, J.A. (1987). Prospective study of 114 consecutive episodes of *Staphylococcus aureus* bacteraemia. *Rev. Infect. Dis*. **9,** 891–897.

Nagar, B., Overduin, M., Ikura, M. and Rini, J.M. (1996). Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* **380,** 360–364.

Nakamura, M.M., Rohling, K.L., Shashaty, M., Lu, H., Tang, Y.W. and Edwards, K.M. (2002). Prevalence of methicillin-resistant *Staphylococcus aureus* nasal carriage in the community pediatric population. *Pediatr. Infect. Dis. J*. **21,** 917–922.

Ninomiya, J., Ito, Y. and Takiuchi, J. (2000). Purification of a protease from a mixture of exfoliative toxin and newborn-mouse epidermis. *Infect. Immun*. **68,** 5044–5049.

Noble, W.C. (1998). Skin bacteriology and the role of *Staphylococcus aureus* in infection. *Br. J. Dermatol*. **139** (Suppl. 53), 9–12.

Noble, W.C., Valkenburg, H.A. and Wolters, C.H.L. (1967). Carriage of *Staphylococcus aureus* in random samples of a normal population. *J. Hyg. (Lond.)* **65,** 567–573.

Novick, R.P. (2000). Pathogenicity factors and their regulation. In: *Gram-positive Pathogens* (eds. V.A. Fischetti, R.P. Novick, J.J. Ferretti, D.A. Portoy and J.I. Rood), pp 392–407. American Society for Microbiology, Washington, D.C.

Novick, R.P. (2003). Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. *Plasmid* **49,** 93–105.

Ochsendorf, F.R., Schofer, H. and Milbradt, R. (1988). Diagnostik des Lyell syndroms: SSSS oder TEN? *Dtsch. Med. Wochenschr*. **133,** 860–863.

Omoe, K., Imanishi, K., Hu, D.L., Kato, H., Takahashi-Omoe, H., Nakane, A., Uchiyama, T. and Shinagawa, K. (2004) Biological properties of staphylococcal enterotoxin-like toxin type R. *Infect. Immun*. **72,** 3664–3667.

Opal, S.M., Johnson-Winegar, A.D. and Cross, A.S. (1988). Staphylococcal scalded skin syndrome in two immunocompetent adults caused by exfoliatin B-producing *Staphylococcus aureus*. *J. Clin. Microbiol*. **26,** 1283–1286.

Paliard, X., West, S.G., Lafferty, J.A., Clements, J.R., Kappler, J.W., Marrack, P. and Kotzin, B.L. (1991). Evidence for the effects of a superantigen in rheumatoid arthritis. *Science* **253,** 325–329.

Parker, M.T., Tomlinson, A.J.H. and Williams, R.E.O. (1955). Impetigo contagiosa: the association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg. Camb*. **53,** 458–473.

Peacock, S.J., Justice, A., Griffiths, D., de Silva, G.D., Kantzanou, M.N., Crook, D., Sleeman, K. and Day, N.P. (2003). Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. *J. Clin. Microbiol*. **41,** 5718–5725.

Pertz, O., Bozic, D., Koch, A.W., Fauser, C., Brancaccio, A. and Engel, J. (1999). A new crystal structure, $Ca^{2+}$ dependence and mutational analysis reveal molecular details of E-cadherin homoassociation. *EMBO J*. **18,** 1738–1747.

Pickard, S., Shankar, G. and Burnham, K. (1994). Langerhan cell depletion by staphylococcal superantigens. *Immunol*. **83,** 568–572.

Piémont, Y. and Monteil, H. (1983). Mise en evidence par electrosynerese des deux serotypes d'exfoliatine produits par *Staphylococcus aureus*. *Ann. Microbiol. (Inst. Pasteur)* **134A,** 169–175.

Pittet, D. and Winzel, R.P. (1995). Nosocomial bloodstream infections: secular trends in rates, mortality, and contribution to total hospital deaths. *Arch. Intern. Med*. **155,** 174–184.

Plano L.R.W., Gutman, D.M., Woischnik, M. and Collins, C.M. (2000). Recombinant *Staphylococcus aureus* exfoliative toxins are not bacterial superantigens. *Infect. Immun*. **68,** 3048–3052.

Plano, L.R. (2004). *Staphylococcus aureus* exfoliative toxins: how they cause disease. *J. Invest. Dermatol*. **122,** 1070–1077.

Plano, L.R.W., Adkins, B., Woischnik, M., Ewing, R. and Collins, C.M. (2001). Toxin levels in serum correlate with the development of staphylococcal scalded skin syndrome in a murine model. *Infect. Immun*. **69,** 5193–5197.

Pollack, S. (1996). Staphylococcal scalded skin syndrome. *Pediatr. Rev*. **17,** 18.

Prasad, G.S., Earhart, C.A., Murray, D.L., Novick, R.P., Schlievert, P.M. and Ohlendorf, D.H. (1993). Structure of toxic shock syndrome toxin-1. *Biochemistry* **32,** 13761–13766.

Prevost, G., Rifai, S., Chaix, L. and Piemont, Y. (1991). Functional evidence that the Ser-195 residue of staphylococcal exfoliative toxin is essential for biological activity. *Infect. Immun*. **59,** 3337–3339.

Rago, J.V., Vath, G.M., Tripp, T.J., Bohach, G.A., Ohlendorf, D.H. and Schlievert, P.M. (2000). Staphylococcal exfoliative toxins cleave alpha- and beta-melanocyte stimulating hormones. *Infect. Immun*. **68,** 2366–2368.

Raymond, J. and Aujard, Y. (2000). Nosocomial infections in pediatric patients: a European, multicenter prospective study. *European Study Group. Infect. Control Hosp. Epidemiol*. **21,** 260–263.

Raymond, J., Bingen, E., Brahimi, N., Bergeret, M., Lepercq, J., Badoual, J. and Gendrel, D. (1997). Staphylococcal scalded skin syndrome in a neonate. *Eur. J. Clin. Microbiol. Infect. Dis*. **16,** 453–454.

Reacher, M.H., Shah, A., Livermore, D.M., Wale, M.C., Graham, C., Johnson, A.P., Heine, H., Monnickendam, M.A., Barker, K.F., James, D. and George, R.C. (2000). Bacteremia and antibiotic resistance of its pathogens reported in England and Wales between 1990 and 1998: trend analysis. *BMJ* **320,** 213–216.

Redpath, M.B., Foster, T.J. and Bailey, C.J. (1991). The role of serine protease active site in the mode of action of epidermolytic toxin of *S. aureus*. *FEMS Microbiol. Lett*. **81,** 151–156.

Rhody, C. (2000). Bacterial infections of the skin. *Prim. Care* **27,** 459–473.

Rogolsky, M., Wiley, B.B., Keyhani, M. and Glasgow, L.A. (1974). Interaction of staphylococcal exfoliative toxin with concanavalin A. *Infect. Immun*. **10,** 1260–1265.

Sakurai, S. and Kondo, I. (1979). A possible receptor substance for staphylococcal exfoliatin isolated from mice. *Jpn. J. Med. Sci. Biol*. **32,** 85–88.

Sakurai, S., Suzuki, H. and Kondo, I. (1987). Cloning of the gene coding for staphylococcal exfoliative toxin A and its expression in *Escherichia coli. FEMS Microbiol. Lett*. **42,** 63–67.

Saloga, J., Enk, A.H., Becker, D., Mohammadzadeh, M., Spieles, S., Bellinghausen, I., Leung, D.Y., Gelfand, E.W. and Knop, J. (1995). Modulation of contact sensitivity responses by bacterial superantigen. *J. Investig. Dermatol*. **105,** 220–224.

Sarai, Y., Nakahara, H., Ishikawa, T., Kondo, I. and Futaki, S. (1977). A bacteriological study on children with staphylococcal toxic epidermal necrolysis in *Japan. Dermatologica* **154,** 161–167.

Sato, H., Matsumori, Y., Tanabe, T., Saito, H., Shimizu, A., Akira, A. and Kawano, J. (1994) A new type of staphylococcal exfoliative toxin isolated from a *Staphylococcus aureus* strain isolated from a horse with phlegmon. *Infect. Immun*. **62,** 3780–3785.

Sato, H., Watanabe, T., Higuchi, K., Teruya, K., Ohtake, A., Murata, Y., Saito, H., Aizawa, C., Danbara, H. and Maehara, N. (2000). Chromosomal and extrachromosomal synthesis of exfoliative toxin from *Staphylococcus hyicus. J Bacteriol*. **182,** 4096–4100.

Schmidt, H. and Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clin. Microbiol. Rev*. **17,** 14–56.

Schrezenmeier, H. and Fleischer, B. (1987). Mitogenic activity of staphylococcal protein A is due to contaminating staphylococcal enterotoxins. *J. Immunol. Methods* **105,** 133–137.

Shapiro, L., Fannon, A.M., Kwong, P.D., Thompson, A., Lehmann, M.S., Grubel, G., Legrand, J.F., Als-Nielsen, J., Colman, D.R. and Hendrickson, W.A. (1995). Structural basis of cell-cell adhesion by cadherins. *Nature* **374,** 327–337.

Sharma, S. and Verma, K.K. (2001). Skin and soft tissue infection. *Indian J. Pediatr*. **68** (Suppl. 3), S 46–50.

Shupp, J.W., Jett, M., Pontzer, C.H. (2002). Identification of a transcytosis epitope on staphylococcal enterotoxins. *Infect. Immun*. **70,** 2178–2186.

Skov, L. and Baadsgaard, O. (1995). Superantigens: do they have a role in skin diseases? *Arch. Dermatol*. **131,** 829–832.

Smith, T.P. and Bailey, C.J. (1990). Activity requirements of epidermolytic toxins from *Staphylococcus aureus* studied by an *in vitro* assay. *Toxicon*. **28,** 675–683.

Stevens, D.L., Tanner, M.H., Winship, J., Swarts, C., Ries, K.M., Schlievert, P.M. and Kaplan, E. (1989). Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. *N. Engl. J. Med.* **321,** 1–7.

Stinnisen, P., Vandevyver, C., Raus, J. and Zhang, J. (1995). Superantigen reactivity of gamma delta cell clones isolated from patients with multiple sclerosis and controls. Cell. Immunol. **166,** 227–235.

Strange, P., Skov, L. and Baadsgaard, O. (1994). Interferon gamma-treated keratinocytes activate T cells in the presence of superantigens: involvement of major histocompatibility complex class II molecules. J. Investig. Dermatol. **102,** 150–154.

Streilein, J.W. (1989). Skin associated lymphoid tissue. In: *Immune Mechanisms in Cutaneous Disease* (ed. D.A. Norris). pp. 73–94. Marcel Dekker Inc., New York.

Stulberg, D.L., Penrod, M.A. and Blatny, R.A. (2002). Common bacterial skin infections. *Am. Fam. Physician* **66,** 119–124.

Sugai, M., Hashimoto, K., Kikuchi, A., Inoue, S., Okumura, H., Matsumoto, K., Goto, Y., Ohgai, H., Moriishi, K. and Syuto, B. (1992). Epidermal cell differentiation inhibitor ADP-ribosylates small GTP-binding proteins and induces hyperplasia of epidermis. *J. Biol. Chem*. **267,** 2600–2604.

Tait, S.C. (1973). The hydrolysis of lipids and esters by *Staphylococcus aureus*. D.Phil. thesis. Paisley College of Technology, Paisley, United Kingdom.

Takiuchi, I., Kawamura, I., Teramoto, T. and Higuchi, D. (1987). Staphylococcal exfoliative toxin induces caseinolytic activity. *J. Infect. Dis.* **156,** 508–509.

Tamura, K., Shan, W.S., Hendrickson, W.A., Colman, D.R. and Shapiro, L. (1998). Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron* **20,** 1153–1163.

Tanabe, T., Sato, H., Kuramoto, M. and Saito, H. (1993). Purification of exfoliative toxin produced by *Staphylococcus hyicus* and its antigenicity. *Infect. Immun*. **61,** 2973–2977.

Tanabe, T., Sato, H., Ueda, K., Chihara, H., Watanabe, T., Nakano, K. Saito, H. and Maehara, N. (1995). Possible receptor for exfoliative toxins produced by *Staphylococcus hyicus* and *Staphylococcus aureus. Infect. Immun*. **63,** 1591–1594.

Taskapan, M.O. and Kumar, P. (2000). Role of staphylococcal superantigens in atopic dermatitis: from colonization to inflammation. *Ann. Allergy Asthma Immunol*. **84,** 3–10.

Terauchi, R., Sato, H., Hasegawa, T., Yamaguchi, T., Aizawa, C. and Maehara, N. (2003). Isolation of exfoliative toxin from *Staphylococcus intermedius* and its local toxicity in dogs. *Vet. Microbiol*. **94,** 19–29.

Tokura, Y., Yagi, J., O'Malley, M., Lewis, J., Takigawa, M., Edelson, R. and Tigelaar, R.E. (1994). Superantigenic staphylococcal exotoxins induce T cell proliferation in the presence of langerhan cells or class II-bearing keratinocytes to produce T cell activating cytokines. *J. Investig. Dermatol*. **102,** 31–38.

Vath, G.M., Earhart, C.A., Monie, D.D., Iandolo, J.J., Schlievert, P.M. and Ohlendorf, D.H. (1999). The crystal structure of exfoliative toxin B: a superantigen with enzymatic activity. *Biochemistry* **38,** 10239–10246.

Vath, G.M., Earhart, C.A., Rago, J.V., Kim, M.H., Bohach, G.A., Schlievert, P.M. and Ohlendorf, D.H. (1997). The structure of the superantigen exfoliative toxin A suggests a novel regulation as a serine protease. *Biochemistry* **36,** 1559–1566.

Verba, V. and Tarkowski, A. (1996). Participation of V beta 4(+)-, V beta 7(+)-, and V beta 11(+)-T lymphocytes in hematogenously acquired *Staphylococcus aureus* nephritis. *Scand. J. Immunol*. **44,** 261–266.

von Eiff, C., Becker, K., Machka, K., Stammer, H. and Peters, G. (2001). Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* **344,** 11–16.

Von Rittershain, G.R. (1878). Die exfoliative Dermatitis jungener senglinge. *Z. Kinderheilkd.* **2,** 3–23.

Weinke, T., Schiller, R., Fehrenbach, F.J. and Pohle, H.D. (1992). Association between *Staphylococcus aureus* nasopharyngeal colonization and septicemia in patients infected with the human immunodeficiency virus. *Eur. J. Clin. Microbiol. Infect. Dis.* **11,** 985–989.

Weinstein, H.J. (1995). The relation between the nasal staphylococcal-carrier state and the incidence of postoperative complications. *N. Engl. J. Med.* **260,** 1303–1308.

Wenzel, R.P. and Perl, T.M. (1995). The significance of nasal carriage of *Staphylococcus aureus* and the incidence of postoperative wound infection. *J. Hosp. Infect.* **31,** 13–24.

White, J., Herman, A., Pullen, A., Kubo, R., Kappler, J. and Marrack, P. (1989). The β-specific superantigen staphylococcal enterotoxin B: stimulation of mature cells and clonal deletion in neonatal mice. *Cell* **56,** 27–35.

Wiley, B.B. and Rogolsky, M.S. (1985). Phospholipase activity associated with electrfocused Staphylococcal exfoliative toxin. In: *The*

*Staphylococci* (ed J. Jeljaszewicz). pp. 295–300. Gustav Fischer Verlag, Stuttgart.

Wiley, B.B., Allman, S., Rogolsky, M., Norden, C.W. and Glasgow, LA. (1974). Staphylococcal scalded skin syndrome: potentiation by immunosuppression in mice: toxin-mediated exfoliation in a healthy adult. *Infect. Immun.* **9,** 636–640.

Willard, D., Monteil, H., Piémont, Y., Assi, R., Messer, J., Lavillaureix, J., Minck, R. and Gauder, R. (1982). L'exfoliatine dans les staphylococcies neonatales. *Nouv. Presse Med*. **11,** 3769–3771.

Wong, G.W., Oppenheimer, S.J., Evans, R.M., Leung, S.S. and Cheng, J.C. (1993). Pyomyositis and staphylococcal scalded skin syndrome. *Acta Paediatr*. **82,** 113–115.

Wuepper, K. D., R. L. Dimond, and D. D. Knutson. (1975). Studies of the mechanism of epidermal injury by a staphylococcal epidermolytic toxin. *J. Investig. Dermatol*. **65,** 191–200.

Yamaguchi, T., Hayashi, T., Takami, H., Nakasone, K., Ohnishi, M., Nakayama, K., Yamada, S., Komatsuzawa, H. and Sugai, M. (2000). Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. *Mol. Microbiol*. **38,** 694–705.

Yamaguchi, T., Hayashi, T., Takami, H., Ohnishi, M., Murata, T., Nakayama, K., Asakawa, K., Ohara, M., Komatsuzawa, H. and Sugai, M. (2001). Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infect Immun*. **69,** 7760–7771.

Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M. and Sugai, M. (2002). Identification of the *Staphylococus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect. Immun*. **70,** 5835–5845.

Yokota, S., Imagawa, T. Kataakura, S. Mitsuda, T. and Arai, T. (1996). Staphylococcal scalded skin syndrome caused by exfoliative toxin B-producing methicillin-resistant *Staphylococcus aureus. Eur. J. Paediatr*. **155,** 722.

Yokote, R., Tokura, Y. Furukawa, F. and Takigawa, M. (1995). Susceptible responsiveness to bacterial superantigens in peripheral blood mononuclear cells from patients with psoriasis. *Arch. Dermatol. Res*. **287,** 443–447.

Zhao, Y.X., Ljungdahl, A. Olsson, T. and Tarkowski, A. (1996). *In situ* hybridization analysis synovial and systemic cytokine messenger RNA expression in superantigen-mediated staphylococcal arthritis. *Arthritis Rheum*. **39,** 959–967.

Zollner, T.M., Wichelhaus, T.A., Hartung, A., Von Mallinckrodt, C., Wagner, T.O., Brade, V. and Kaufmann, R. (2000). Colonization with superantigen-producing *Staphylococcus aureus* is associated with increased severity of atopic dermatitis. *Clin. Exp. Allergy* **30,** 994–1000.

CHAPTER

# 57

# Bacterial toxins as food poisons

*Per Einar Granum*

## INTRODUCTION

Food- and waterborne illness is a common, distressing, and sometimes life-threatening problem for people worldwide. People in developing countries suffer the most from these diseases. A significant proportion of cases of the estimated 1.5 billion episodes/year of diarrhea in children under five, including three million deaths, are of food- and waterborne origin. In many industrialized countries, despite all precautions, the incidence of foodborne infection has increased in recent years, although some have managed to reduce the numbers over the last few years. Surveys indicate that 5–10% of the population is affected annually, and in addition to the human suffering, food poisoning causes substantial economic loss. In the USA, costs are estimated to be US $5.8–10.7 billion/year, and between £231–331 million in England and Wales (Sockett and Todd, 2000). Although the two figures are not completely comparable, they indicate that food poisoning costs society about two to three times as much in the USA as in England and Wales after population adjustments. The ratio for bacterial infections were however 1.4:1 in 2000 between USA and England and Wales (Adak *et al.*, 2002). One single outbreak of cholera in Peru costs US $495 million in lost fish exports and in decreased tourism and local food sales (Sockett and Todd, 2000; WHO, 1997). Unfortunately, reliable/comparable figures from the developing world are not available.

The bacteria responsible for causing food poisoning have managed to adapt to the new niches presented to them with modern food processing. Much of the adaptation is through assimilation of new genes, including toxin- and invasion-related genes. The virulence factors that are responsible for the direct action on host tissues, and thereby development of disease, are dependent on the physical and genetic background of both the bacteria and the host. The toxins are, for some of the food poisoning diseases, the only cause of the symptoms but may for others only increase the severity of the disease. Many of the toxins described here are on mobile elements (plasmids, phages, and transposons), and can conceivably be transferred to bacteria that have not yet been identified as potential food pathogens. The presence of identical (or very similar) genes in different species of bacteria has caused confusion in terminology of the proteins and of the bacteria.

## THE FOOD-POISONING BACTERIA

There are a limited number of bacteria that are able to cause food poisoning, and they act through either intoxications or infections. For some of these species, the toxin(s) may be the only cause of the symptoms, while for others, the toxin(s) play(s) a moderate to minor role in pathogenicity. The role of the toxins along with the mechanisms of pathogenicity make it possible to divide the food-poisoning bacteria into five different groups according to the criteria given in Table 58.1. These five different groups are also illustrated in Figure 57.1. Group 1 contains the bacteria causing foodborne intoxications (toxins preformed in foods), while the remaining four groups comprise the species that have the ability to give us foodborne infections. For foodborne intoxications, the bacteria may or may not be present in the food at the time of consumption,

**TABLE 57.1** The different groups of bacteria causing food poisoning divided according to pathogenicity mechanisms

| Group[a] | Mechanisms of pathogenicity |
|---|---|
| 1 | Enterotoxin or neurotoxin preformed in foods |
| 2 | Enterotoxin produced in the intestine without bacterial adherence to epithelial cells |
| 3 | Enterotoxin produced in the intestine after bacterial adherence to epithelial cells |
| 4 | Bacterial invasion usually localized to the epithelium and intestinal immune system, with or without toxin production |
| 5 | Systemic infections |

[a]Bacterial intoxications: group 1, bacterial infections: group 2–5

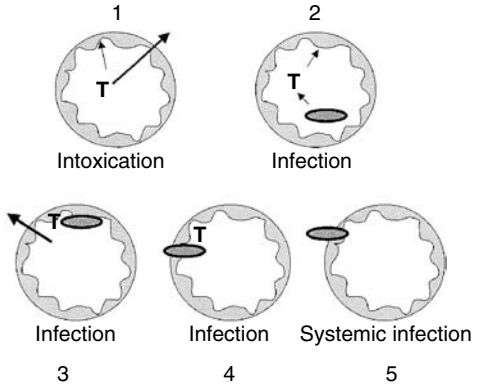



**FIGURE 57.1** Schematic illustration of the five different groups of foodborne pathogens. The circles indicate a cross-section of the intestine. The white area is the intestinal lumen, the dark gray a bacterium, and the **T** the toxin produced (see also the description in Table 57.1). The arrows pointing out of the intestine are for the botulinum toxin (group 1) and for the Shiga toxin of group 3.

and the bacteria can be killed by heat while the toxin survives (*S. aureus* and *B. cereus* emetic type). Taking a closer look at the species belonging to group 2 and 3 (Tables 57.1, 57.2, and Figure 57.1), we see that although they are by definition infections, the symptoms are almost entirely caused by toxin production in the host. In contrast, for the members of groups 4 and 5, which usually cause the most severe infections (frequently with fever), the toxins play a less important role in pathogenicity, mainly because these bacteria are able to invade the hosts cells, and the bacteria in group 5 cause systemic infections. From our present knowledge, it appears that protein toxins of the type discussed in this chapter do not play a direct role in virulence/pathogenesis for the bacteria in group 5, and these bacteria will not be discussed in this chapter.

Most of the foodborne diseases have been well characterized, and it is possible to summarize the knowledge on infective dose, incubation time, symptoms, and duration as seen in Table 57.2. For the intoxications caused by the members of group 1, the toxins are always preformed in the food (with an exception for infant botulism). The next group of bacteria produce the toxins (enterotoxins) in the small intestine of the host, but normally without any interaction with the host epithelial cells. Even for the members of group 3, the bacteria has little effect on the host, although colonization of the epithelial cells in usually necessary prior to enterotoxin formation. For the members of the first three groups, although the diseases might be severe (i.e., *C. botulinum*, EHEC, and *V. cholera*), fever is not a common symptom and is never high. In contrast, fever is usually a major symptom for infections caused by the members of groups 4 and 5 because of the bacteria's direct interaction and invasion of the host.

## THE TOXINS

The toxins involved in bacterial food poisoning mainly belong to three different groups of toxins: emetic toxins, neurotoxins, and enterotoxins (Table 57.3). Emetic toxins cause vomiting by binding specific receptors in the duodenum. Neurotoxins are proteins or small chemical substances (not relevant in this context) that act on the nervous system. Enterotoxins are defined as protein toxins that are active in the intestine and causediarrhea (fluid accumulation in loop tests). Enterotoxins can be divided into two groups: cytotoxic enterotoxins, which disrupt the cell membrane or other vital functions in the cell, thus causing cell death; and cytotonic enterotoxins, which enter the epithelial cell and cause diarrhea without direct membrane disruption or cell death. Table 57.4 shows some characteristics of the toxins of the different food-poisoning bacteria. Several of these toxins are described in detail in other chapters in this book, and will only be treated briefly here.

### *Staphylococcus aureus* enterotoxin

*Staphylococcus aureus* is a Gram-positive cocci, and the main reservoir is humans and warm-blooded animals. About 50–70% of strains have the ability to produce the *S. aureus* enterotoxins (SE) that are the cause of one of the most common types of food poisoning in the Western world. The enterotoxin is preformed in foods stored above 15–18°C, and it usually takes more than 3 h to produce enough enterotoxin to cause food poisoning. As little as 100–200 ng may cause emesis in man (Jablonski and Bohach, 2001). The enterotoxins are specific to primates and are also potent mitogenic superantigens (Marrack and Kappler, 1990). They consist of a family of seven different but related toxins (Alouf *et al*.,

**TABLE 57.2** The most important food poisoning organisms

| Species | Infective dose | Incubation time | Symptoms* | Duration |
|---|---|---|---|---|
| **Intoxications** | | | | |
| **Group 1** | | | | |
| *Staphylococcus aureus* | toxin | 1–6 h | N A V (D F) | 8–24 h |
| *Bacillus cereus* (emetic) | toxin | 1–6 h | N V | 6–24 h |
| *Clostridium botulinum* | toxin | 12–72 h | neurological | days–months |
| **Infections** | | | | |
| **Group 2** | | | | |
| *Bacillus cereus* (diarrheal type) | $10^5$–$10^7$ | 6–12 h | A D | 12–24 h |
| *Clostridium perfringens* | $10^7$–$10^8$ | 8–16 h | A D N(F) | 16–24 h |
| **Group 3** | | | | |
| *Aeromonas* spp. | $10^6$–$10^8$ | 6–48 h | D A (F) | 24–28h |
| *Escherichia coli* | | | | |
| • ETEC (ST) | $10^5$–$10^8$ | 16–48 h | D (A V F) | 1–2 days |
| • ETEC (LT) | $10^5$–$10^7$ | 16–48 h | D (A V F) | 1–3 days |
| • EHEC (O157:H7) | 10 | 1–7 days | D A B (H) | days–weeks |
| *Vibrio cholerae* | $10^8$ | 2–5 days | D A (V) | 4–6 days |
| *Vibrio parahaemolyticus* | $10^5$–$10^7$ | 3–76 h | D A (N V F) | 3–7 days |
| **Group 4** | | | | |
| *Campylobacter jejuni/coli* | $\geq 10^3$ | 3–8 days | F A D B | weeks |
| *Salmonella* spp (non-typhoid) | $10^3$–$10^6$ | 6–72 h | D A F (V H) | 2–7 days |
| *Shigella* spp. | $10^2$–$10^5$ | 1–7 days | A F D B (H N V) | days–weeks |
| *Yersinia enterocolitica* | $10^6$–$10^7$ | 3–5 days | F D A (V H) | weeks |
| **Group 5** | | | | |
| *Listeria monocytogenes* | $10^7$–$10^8$ | days | systemic | weeks |
| *Salmonella typhi* | 1–$10^2$ | 10–21 days | systemic | weeks |
| *Salmonella paratyphi* | 1–$10^2$ | 10–21 days | systemic | weeks |

* A: Abdominal pain
B: Bloody diarrhea
D: Diarrhea
F: Fever
H: Headache
N: Nausea
V: Vomiting

[a]Symptoms in the order they usually appear

1991). Similar toxins are in rare cases found in other *Staphylococcus* spp. These toxins cause vomiting, yet they are called enterotoxins because of their ability to produce fluid accumulation in ileal loop tests. The SE also cause diarrhea, but not a severe type, and probably only at higher concentrations than needed for the cause of emesis. It has recently been shown that drugs blocking receptors for serotonin completely ablated the vomiting, diarrhea, and prostration induced by SEA or SEB in piglets (Hammamieh *et al*., 2003). Although these toxins are proteins (MW 26–28 kDa), the active part of the molecule is not influenced by heat treatment, so even autoclavation is not sufficient to reduce activity of preformed SE. Thus SE may be found in sterile heat-treated foods where it has been produced prior to heat treatment, i.e., canned foods.

**TABLE 57.3** Different types of toxins involved in food poisoning

| Types of toxins |
|---|
| Emetic toxins |
| Neurotoxins |
| Enterotoxins |
| Cytotoxic enterotoxins (kill cells) |
| Membrane active |
| Enzymatic active |
| Glycosidase toxins |
| Cytotonic enterotoxins (modify cells from inside) |
| Ribosylase toxins |

## *Clostridium botulinum* neurotoxin

Botulism is a rare but often fatal disease, caused by the spore-forming bacterium *Clostridium botulinum*. The main reservoir for this bacterium is soil. The different types of *C. botulinum* vary in potency and geographical locations. In Argentina, Brazil, China, and western parts of the USA, where the highly potent type A is the most frequently isolated type, fatality rates are higher than countries where other types are prevalent (Austin, 2001). *Clostridium botulinum* produces seven different neurotoxins (Hauschild, 1989). *C. tetani* neurotoxin is related, but not involved in food poisoning. Also, other *Clostridium* species may produce neurotoxins of the type found in *C. botulinum*. All the botulinum toxins are now known to be $Zn^{++}$ dependent endopeptidases, which split three different neuronal proteins at specific

**TABLE 57.4** Characteristics of toxins involved in food poisoning produced by different bacteria

| Species | Number of toxins | Toxin type* | Heat labile (L) stable (S) | Receptor | Mode of action | Enzyme activity |
|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | Many different types | Emetic and CnEnt, protein | S | TCRVβ T-cells | Emesis: via nervus vagus and sympaticus | ? |
| *Bacillus cereus* (emetic) | 1 | Emetic cyclic peptide | S | $5\text{-HT}_3$ | Emesis: via nervus vagus | No |
| *Clostridium botulinum* | 7 (type A – G) closely related | Neurotoxin, protein | L | GT1b or GD1a | Split presynaptic membrane proteins | Zn++ dependent endopeptidase |
| *Bacillus cereus* (diarrheal type) | 3 (Hbl, Nhe and CytK) | CtEnt protein | L | Unknown | Membrane disruption | ? |
| *Clostridium perfringens* type A | 1 (CPE) | CtEnt protein | L | claudins 50 kDa protein | Membrane disruption | No |
| *Aeromonas* spp. | 1 (aerolysin) | CtEnt | L | GPI anchor | Membrane disruption | No |
| | many other types | CtEnt and CnEnt | L and S | ? | ? | ? |
| *Escherichia coli* | | | | | | |
| • ETEC (ST) | several | CnEnt, peptide | S | | cGMP accum. | ? |
| • ETEC (LT) | 1 | CnEnt, protein | L | GM1 | cAMP accum. | ADP-ribosylase |
| • EHEC (O157:H7) | 2 | CtEnt, protein | L | Gb3 | Inhib. of protein synthesis | *N*-glycosidase |
| *Vibrio cholerae* | 1 (CT) | CnEnt, protein | L | GM1 | cAMP accum. | ADP-ribosylase |
| *Vibrio parahaemolyticus* | 2 (TDH/TRH) | CtEnt, protein | S | GM2/GM1 | Membrane disruption Ca++ signal transduction | ? |
| *Campylobacter jejuni/coli* | 1 (additional types) | CnEnt, protein | L | GM1 | cAMP accum. | ADP-ribosylase |
| *Salmonella* spp | 2 | CnEnt, protein | L | GM1 | cAMP accum. | ADP-ribosylase |
| | | CtEnt, protein | L | ? | Inhib. of protein synthesis | *N*-glycosidase |
| *Shigella* spp. | 1 | CtEnt, protein | L | Gb3 | Inhib. of protein synthesis | *N*-glycosidase |
| *Yersinia enterocolitica* | 1 (additional types) | CnEnt, peptide | S | M-cells (uncertain) | cGMP accum. | ? |

*Ent-enterotoxin Cn-cytotonic Ct-cytotoxic

target sites (Montecucco and Schiavo, 1994). In contrast to *S. aureus* enterotoxins, the botulinum toxins are heat labile and are rapidly reduced in activity at 60°C (Hauschild, 1989). As expected, these toxins are usually produced in non-heat-treated foods stored above 3–10°C (differ for proteolytic and non-proteolytic strains) for longer periods of time, and only under anaerobic conditions. Occasionally recontaminations of cooked food products have been involved. As seen from Figure 57.1, the neurotoxins are not active in the gut, but only after transport into the blood, and further to the target cells. In infant botulism (only in children under one year of age), *C. botulinum* spores adhere to epithelial cells in the intestine, germinate, and colonize the intestine and produce the toxin there. Thus, this type of botulism belongs to group 3 (Table 57.1, Figure 57.1).

## *Bacillus cereus* toxins

*Bacillus cereus* is a Gram-positive, spore-forming, motile, facultative anaerobic rod. *B. cereus* causes two different types of food poisoning: the diarrheal type and the emetic type. The diarrheal type of food poisoning is caused by at least three different types of enterotoxins (Granum 2001), produced during vegetative growth of *B. cereus* in the small intestine (Granum, 1994), while the emetic toxin is produced by growing cells in the food (Kramer and Gilbert, 1989). The closely related *B. thuringiensis* is reported to produce enterotoxins (Jackson *et al*., 1995; Ray, 1991), and this could potentially cause serious problems, as spraying of this organism to protect crops against insect attacks has become common in several countries. There has been at least one confirmed *B. thuringiensis* outbreak of food poisoning (Jackson *et al*., 1995).

The dominating type of disease caused by *B. cereus* differs from country to country. In Japan the emetic type is reported about 10 times more frequently than the diarrheal type (Kramer and Gilbert, 1989), while in Europe and North America the diarrheal type is the most frequently reported (Kramer and Gilbert, 1989). The emetic toxin results in vomiting, while the diarrheal type, caused by enterotoxins, produces diarrhea (Granum, 2001). In a small number of cases, both types of symptoms are recorded (Kramer and Gilbert, 1989), probably due to production of both types of toxins. Counts ranging from $10^3$–$10^9$/g (or ml) *B. cereus* (Granum, 2001) have been reported in the incriminated foods after food poisoning, giving total infective doses ranging from about $10^5$–$10^{11}$. Partly due to the large differences in the amount of enterotoxin produced by different strains (Granum, 2001), the total infective dose seems to vary between about $10^5$–$10^8$ viable cells or spores. Thus, any food containing more than $10^3$ *B. cereus*/g cannot be considered completely safe for consumption.

The emetic toxin causes emesis (vomiting) only and its structure remained a mystery as long as the only detection system involved living primates (Kramer and Gilbert, 1989). The emetic toxin cereulide consists of a ring structure of three repeats of four amino and/or oxy-acids: $[D\text{-}O\text{-Leu-D-Ala-L-}O\text{-Val-L-Val}]_3$. This ring structure (dodecadepsipeptide) has a molecular mass of 1.2 kDa, and is chemically closely related to the potassium ionophore valinomycin (Agata *et al*., 1994). The emetic toxin is resistant to heat, pH, and proteolysis but is not antigenic (Kramer and Gilbert, 1989). The biosynthesis of the emetic toxin is through a large enzyme complex called a non-ribosomal peptide synthetase (NRPS) (Ehling-Schulz *et al*., 2005). It has been shown that it stimulates the vagus afferent through binding to the 5-$HT_3$ receptor (Agata *et al*., 1995).

*B. cereus* produces two different three-component enterotoxins (Beecher and Wong, 1997; Lund and Granum, 1997; Beecher and Wong, 1994; Beecher *et al*., 1995; Lund and Granum, 1996). They are called the hemolysin BL (Hbl) and the non-hemolytic enterotoxin (Nhe). Also a third one-component enterotoxin, cytotoxin K, has been described (Lund *et al*., 2000). This protein has a molecular mass of about 34 kDa and belongs to a family of β-barrel channel-forming toxins (Montoya and Gouaux, 2003). All these toxins are produced during the vegetative growth of *B. cereus* in the small intestine and are membrane active (Table 57.4).

## *Clostridium perfringens* enterotoxin

*Clostridium perfringens* are ubiquitous spore-forming, anaerobic, Gram positive, non-motile rods that grow well between 20 and 50°C (Hatheway, 1990). They are commonly found as a part of the intestinal flora of mammals. *C. perfringens* can produce at least 13 different toxins, though each individual isolate only produces a subset of these. The production of the four major lethal toxins is used to type isolates (A–E). The enterotoxin gene (*cpe*) has been found in all types. There are two major types of food poisoning, a diarrheal type caused by the *Clostridium perfringens* enterotoxin (CPE) and necrotic enteritis mainly caused by the β-toxin (β-barrel channel-forming toxin) in Type C strains.

*C. perfringens* type C can be involved in necrotic enteritis, known in Germany as "Darmbrand" and in New Guinea as "pig-bel." The symptoms are mainly caused by the β-toxin, although δ-toxin (hemolysin) and θ-toxin (perfringolysin O) contribute to the disease, which can be quite serious and has a mortality rate of 15–25%, even with treatment. The toxin is

trypsin sensitive and is normally inactivated by stomach enzymes. The disease is associated with individuals who have a low level of proteolytic enzymes in their intestinal tracts, most often caused by a low intake of protein. In New Guinea, pig-bel outbreaks coincided with traditional festivals where large amounts of spit-grilled pork were consumed while their staple diet, sweet potatoes, contains a trypsin inhibitor (Granum, 1990). The risk of *C. perfringens* type C food poisoning is minimal for healthy individuals with normal levels of proteolytic enzymes. Although the disease is not common, the danger of this type of food poisoning is real in immunocompromised individuals on special (vegetarian) diets. A relatively mild type of diarrhea is the most common form of food poisoning caused by *C. perfringens* and is one of the most common forms of human gastrointestinal illness in industrialized nations (McClane, 2001; Granum and Brynestad, 2002). Although the enterotoxin gene (*cpe*) has been found in all types of *C. perfringens*, only type A has been associated with CPE that caused food poisoning. After ingestion of food contaminated with large numbers (about $10^8$) of cells, CPE is produced by the bacterium during sporulation in the intestine and released upon lysis of the mother cell. CPE then binds to a protein receptor, after which a pore is formed, resulting in altered membrane permeability, which causes diarrhea.

CPE is a single, 319 amino acid polypeptide with a molecular weight of 35, 317 Da and an isoelectric point of 4.3 and is heat and pH labile (Kokai-Kun *et al*., 1997b). There is no significant homology to any known proteins. A two-domain structure has been proposed where the N-terminal is necessary for insertion/cytotoxicity and the C-terminal end (aa 290–319) contains the binding region, with specific binding necessary for toxicity (Kokai-Kun and McClane, 1997a). The binding also causes a change in secondary structure from mainly β-sheet to mainly α-helix (Granum and Harbitz, 1985). The removal of the first 25 to 34 aa increases activity (Granum *et al*., 1981; Granum and Richardson, 1991), and the first 44 aa can be removed without loss of activity (Kokai-Kun and McClane, 1997a). The observation that strains could lose the ability to produce enterotoxin and the fact that only 5–10% of naturally isolated strains carried *cpe* enterotoxin suggested that *cpe* was carried on some sort of mobile element. This possibility was confirmed when it was found that *cpe* is on a transposon, Tn*5565*, integrated between two housekeeping genes in human food poisoning strains (Brynestad *et al*., 1997). In animal isolates, the gene is carried on large plasmids with some strain-to-strain variation in the genetic structure (Cornillot *et al*., 1995; Miyamoto *et al*., 2002).

## *Aeromonas* spp. enterotoxin

Many different *Aeromonas* spp. have been suspected to cause gastroenteritis. They are Gram-negative motile rods, and the main reservoir is water. *Aeromonas* spp. have been suspected to be the cause of water- and foodborne (mainly seafood) gastroenteritis (Kirov, 2001), and outbreaks have been reported by several authors (Kirov, 2001). Three species, *A. hydrophila*, *A. caviae*, and *A. veronii* biovar sobria, have been suggested as a cause of human gastroenteritis (Kirov, 2001; Deohar *et al*., 1991). *Aeromonas* spp. is a potential serious food problem, as many of them can grow at refrigeration temperatures, at pH values from 4–10, under most atmospheres used to prevent bacterial growth in foods, and in the presence of high salt concentration (Kirov, 2001; Kirov, 1993). A variety of virulence factors and potential enterotoxins have been characterized (Kirov, 2001), and some strains have been shown to invade epithelial cells (Lawson *et al*., 1985; Watson *et al*., 1985).

The aerolysin and other hemolysins with closely related sequences seem to be the best candidates for the major enterotoxin (Granum *et al*., 1998; Kirov, 2001). These hemolysins are able to disrupt the membranes in epithelial cells and thereby cause diarrhea (Granum *et al*. 1998). It is not unlikely that more than one enterotoxin may be involved in food poisoning of humans, but addressing this question needs much more research, preferably on strains that have been involved in food poisoning. Although involvement of *Aeromonas* spp. in food poisoning is still controversial, an increasing number of papers point in the direction of direct involvement. The number of potential enterotoxins that has been suggested during the past few years (Kirov, 2001) may show the diversity of the different *Aeromonas* spp. strains. They are pathogenic to a variety of different animal species living under very different conditions (from fish to man).

## *Escherichia coli* enterotoxins

*Escherichia coli* is a Gram-negative rod with the intestine of warm-blooded animals as the main reservoir. The majority of the *E. coli* are non-pathogenic; still, there are at least five different types of *E. coli* that can cause food poisoning: enterotoxic (ETEC: two toxin types, ST and LT), enterohemorragic (EHEC: Shiga toxins), enteroinvasive (EIEC), enteropatogenic (EPEC), and enteroaggregative (EAaggEC) (Nataro and Kaper, 1998). The first two types produce enterotoxins (Table 57.2 and 57.4). Enterotoxic *E. coli* are probably the most common cause of travelers' diarrhea, and are an important cause of food poisoning

throughout the world. The main *E. coli* concern for the food industry is EHEC, because of the low infective dose (Table 57.2) and the severity of the disease, especially for the infirm and children, who are susceptible to the development of kidney failure (hemolytic-uremic syndrome: HUS).

Both types of ETEC are relatively mild diseases, and the symptoms are caused only by the enterotoxins. The heat-labile enterotoxin (LT) is among the best-characterized enterotoxins, and it is very similar to the cholera toxins (and an identical mode of action). This toxin consists of six subunits (5B and 1 A) and is an ADP-ribosylating toxin (Table 57.3). The heat-stabile toxins are peptides/small proteins with molecular mass below 10 kDa (18–72 amino acids) and act by activating guanyl cyclase and increasing cyclic GMP. The Shiga toxins produced by EHEC (O157:H7, among other serotypes), which are also found in other related bacteria, are closely related to the Shigella toxin, and with an identical mechanism of action (Table 57.3). There are two types of *E. coli* Shiga toxins (STI and STII), and strains can produce both or only one of them. The Shiga and Shigella toxins also consist of six subunits (5B and 1A), and the enzymatically active A unit is active after entering the target cells, where it modifies the ribosomes (glycosidase, Table 57.3). The genes are phage mediated and seem to be transferred from one bacteria to another. It is almost impossible for the food industry to control the problem of the presence of these types of *E. coli*, as all meat is a potential source of infection, and thus a specific problem in ground meat products.

## *Vibrio* enterotoxins

*Vibrio* spp. are motile, faculatively anaerobic, Gram-negative rods, where eight of the species are known food-associated pathogens. *V. cholera* and *V. parahaemolyticus* are the most common and have the best characterized virulence mechanisms, and are the only two included here. *Vibrio* spp. are found in estuarine waters throughout the world. They can be found in essentially all seafood and raw, undercooked, or recontaminated seafood are the major sources of food poisoning. *Vibrio* has been shown to survive for extended periods of time at refrigeration temperatures and can grow rapidly at room temperature (non-refrigeration) (Oliver and Kaper, 2001).

*Vibrio cholera* can cause an explosive, potentially fatal dehydrating diarrhea caused by ingestion of serotypes (01/0139) that carry the cholera enterotoxin (CT). The majority of infections involving *V. cholera* 01 are mild, and volunteer studies showed large differences in the susceptibility between individuals. Strains without CT can, in some cases, still cause diarrhea, but without the typical "rice water" stools. Additional toxins such as Zot, Ace, hemolysin/cytolysin are suspected to be involved in the pathogenesis of the disease. Genetic factors other than CT would seem to be important, as other bacteria, such as *E. coli*, carry a gene coding for a toxin comparable to CT, but these bacteria have not been shown to give cholera-like symptoms (Oliver and Kaper, 2001). The main source of infection is contaminated water, but also seafood has been involved.

*Vibrio parahaemolyticus* can cause a disease, mainly involving diarrhea, that is linked to the ability to produce the homo dimeric thermostable direct hemolysin (TDH) or the similar hemolysin TRH (Oliver and Kaper, 2001). However, Park *et al*. (2004) have just constructed *tdh*-deletion mutants by homologous recombination and analyzed their phenotypes. Although the deletion of both copies of *tdh* completely abolished the hemolytic activity of the wild-type strain, the deletion did not affect the cytotoxicity to HeLa cells. Enterotoxicity, assayed by the rabbit ileal loop test, was lowered by *tdh* deletion, but the mutant still showed partial fluid accumulation in rabbit intestine. These results indicate that the cytotoxicity and enterotoxicity of TDH-producing *V. parahaemolyticus* are not explained by TDH alone, and unknown virulence factor(s) could be involved in these pathogenic activities (Park *et al*., 2004). TDH is only partially inactivated at 100°C for 30 min. at pH 6.0. TDH produces edma, erythema, induration in skin, and lyses erythrocytes from many animals, excepting horses. More relevant for food poisoning is the ability to alter transport in the intestinal tract and thereby induce diarrhea. Studies using Ussing chambers demonstrated that TDH induces intestinal chloride ion secretion with the ganglioside GT1 or GT1b as the cellular receptor. TDH uses $Ca^{2+}$ as an intercellular second messenger, instead of cAMP or cGMP, and is the first bacterial enterotoxin where changes in intercellular calcium and secretory activity have been shown to be directly linked (Oliver and Kaper, 2001). However, the induction in a human embryonic cell line of a $Ca^{2+}$-independent cytotoxicity due to the hemolysin has also been reported (Tang *et al*., 1995). There is however no doubt that TDH is a membrane active toxin with the ability to form pores in phospholipid bilayers (Hardy *et al*., 2004). The main source of food poisoning is seafood from warm seawater.

## *Campylobacter jejuni/coli* enterotoxins

*Campylobacter jejuni* and *C. coli* are Gram-negative microaeropholic S-shaped motile spiral rods only growing between 30°C and 47°C. They can survive at 4°C for extended periods, and have a viable but not

culturable coccoid form. Reservoirs include water, domestic animals (notably poultry) and pets, and wild birds. Most outbreaks occur in the summer months. Camphylobacteriosis has been the most frequent cause of foodborne outbreaks in some European countries in the nineties (Wassenaar, 1997). Suspected food sources include raw milk, poultry, eggs, beef, and water. Even though *Campylobacter* spp. are susceptible to low pH, heat, and don't grow at low temperatures, the low dosage needed to cause sickness makes it difficult to avoid in food, and it will probably continue to be one of the most common food poisoning agents in industrialized nations. Patients can be unsymptomatic to severely ill. Symptoms include fever, cramping, and diarrhea, with or without leukocytes, that last several days to over a week. Infections are usually self-limiting, but extraintestinal infections and sequelae do occur, including bacteremia, reactive arthritis, and Guillain-Barré syndrome (GBS) (Nachamkin, 2001).

*C. jejuni* has been reported to produce a toxin with cholera-like enterotoxin activity elevating cAMP levels in rabbit ileal loop tests. However, genetic probing using CT probes proved negative, indicating that the sequence must be quite different. Shiga toxins, cytolethal distending toxin, and hepatoxins have also been described. Genetic studies have been difficult to perform in *Campylobacter*, and the lack of suitable animal models has hindered the study of the contribution of the different toxins in pathogenicity (Wassenaar, 1997). The wide range of symptoms seen during *Campylobacter* spp. infections suggests that different strains of *Campylobacter* spp. carry one or a number of different virulence genes, and the severity of the sickness is a result of the expression of the combination of genes found in the infecting bacteria (Poly *et al*., 2004).

## *Salmonella* spp. enterotoxin

*Salmonella* spp. is one of the most common food poisoning organisms in industrialized nations. They are resilient organisms that adapt to extreme environmental conditions, and there are strains that can grow at 54˚C, and others that can grow at 2–4˚C and tolerate pH values from 6.0 to 9.0. These bacteria are a major problem in poultry and egg products and other meat products. Salmonella infections can cause sickness ranging from uncomplicated diarrhea to serious systemic infections. The bacteria has a number of genes that facilitate the attachment to and invasion of host cells, and attachment is necessary for pathogenesis. Although many of the details of invasion of the host cells are known, the contribution of toxins produced by the bacteria in the total pathogenicity picture has not been completely elucidated (Bailey and Maurer, 2001).

Although the enterotoxin is not necessary for development of salmonellosis, it contributes to the disease. One enterotoxin is a thermolabile protein encoded by a 6.3kb operon (*stx*), which encodes three genes. The toxin increases the level of cAMP in cells, which leads to fluid exsorption and to diarrhea. Although genetic studies show that STX and cholera toxin are not closely related, functional studies show that the toxins share a similar mode of action with A and B components, and probably use the same GM1 ganglioside receptor (D'Aoust *et al*., 2001). *Salmonella* also produce a thermolabile cytotoxic protein localized in the bacterial outer membrane. This cytotoxin inhibits protein synthesis, apparently using the same mechanisms as Shiga toxins, although genetic studies show that the toxins are not identical (D'Aoust *et al*., 2001).

## *Shigella* enterotoxin

*Shigella* spp. are Gram-negative, non-motile rods, which are the cause of bacillary dysentery (a human-specific disease). The clinical picture ranges from watery diarrhea to severe dysentery (bloody diarrhea), and complications such as reactive arthritis and kidney failure (HUS) are seen. The infective dose is very low, and that facilitates the infectious spread of the bacteria through food and water. Most of the foodborne incidents are caused by infected food handlers, but the largest outbreaks are after natural or man-made disasters (war) where sanitation and water supply lines are broken down. Attachment and invasion genes are the primary virulence factors, while the production of Shiga toxins (glycosidase, Tables 57.3 and 57.4) seems to play a role in the progression of the disease, as dysentery is not seen in strains without the toxin (Lampel and Maurelli, 2001). The Shiga toxin (Stx) region of *Shigella dysenteriae* 1 lies on a defective prophage homologous to lambdoid bacteriophages in Stx-producing *Escherichia coli* (Greco *et al*., 2004). The Shigella toxin has the same structure and mechanism of action as the Shiga toxins of EHEC.

## *Yersinia enterocolitica* enterotoxin

*Yersinia* is a Gram-negative, facultative anaerobe rod that can grow between 0 and 44˚C. Watery to mucoid diarrhea is the most common symptom, while bacteremia and reactive arthritis are the most common complications. It is not a common form of food poisoning, and is most often seen in northern climates. Pigs are often carriers of *Yersinia*, but raw milk is the most commonly implicated food source. As with *Salmonella* and *Shigella*, invasiveness is the major virulence factor, and these genes are homologous in all three bacteria.

Most strains of *Yersinia* secrete a heat-stable enterotoxin, Yst (three different types), which is homologous to the heat-stable toxins from enterotoxigenic *E. coli* and *Vibrio cholera* non-01. The mechanism of action is elevation of intercellular cGMP levels, which affects the fluid transport pathways and results in diarrhea. The role of the toxin here, as in other invasive bacteria, is uncertain, but it appears that toxins can either worsen the clinical symptoms or assist in the attachment to the host cell (Robins-Browne, 2001). The Yst-b has recently been described as the major contributor to diarrhea produced by biotype 1A strains of *Y. enterocolitica* (Singh and Virdi, 2004).

## CONCLUSION

Only a few species of all known bacteria are involved in foodborne illness. It is not sufficient for bacteria to possess genes that can encode for toxins or other virulence factors. The bacteria must also be able to survive one or a number of detrimental and changing environmental factors, including dehydration, heat, cold, low pH values (stomach), competing flora, and other intestinal defense mechanisms. There are many bacteria that carry the same or equivalent virulence genes as bacteria known to cause foodborne illness that have not yet been fully recognized as cause of a human gastroenteritis, i.e., *Bacteroides fragilis* and *Citrobacter* spp. The modes of action for different groups of toxins are becoming clear, but the full importance of these toxins in the clinical picture is still somewhat unclear in all but the first two groups of toxins and some members of group 3 (Tables 57.1, 57.2, and 57.4). Even in these groups, genetic characterization of the different toxins and their prevalence among the strains of the species has just started. Equivalent toxins in different, and even the same, bacterial species can give very different symptoms, apparently due to the genetic background in which the toxins are found. It would be beneficial to the field if the nomenclature of the protein toxins (Granum *et al*., 1995) and the tests used to determine their action, preferably using available cell lines such as Vero, Caco, and HeLa, were standardized. Research on the role of toxins, their movement between bacteria, and regulation of expression is needed in order to effectively monitor and prevent unwanted bacteria in our food, and possibly prevent the rise of new pathogens.

## REFERENCES


Adak, G.K., Long, S.M. and O'Brien, SJ. (2002) Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut*, **51,** 832–841.

Agata, N., Ohta, M. Mori, M. and Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiol. Lett*. **129,** 17–20.

Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I. and Isobe, M. (1994). A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. *FEMS Microbiol. Lett*. **121,** 31–34.

Alouf, J.E., Knöll, H. and Köhler, W. (1991). The family of mitogenic, shock-inducing, and superantigenic toxins from staphylococci and streptococci. In: *Sourcebook of Bacterial Protein Toxins* (eds. Alouf, J.E. and Freer, J.H.), pp. 367–414. Acedemic Press, London.

Austin, J. W. (2001). *Clostridium botulinum*. In: *Food Microbiology: Fundamentals and Frontiers*, 2nd ed. (eds. M. Doyle, L. Beuchat and T. Montville), pp. 329–350. ASM press, Washington D.C.

Beecher, D.J. and Wong, A.C.L. (1994). Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infect. Immun*. **62,** 980–986.

Beecher, D.J. and Wong, A.C.L. (1997). Tripartite hemolysin BL from *Bacillus cereus*. Hemolytic analysis of component interaction and model for its characteristic paradoxical zone phenomenon. *J. Biol. Chem*. **272,** 233–239.

Beecher, D.J., Schoeni, J.L. and Wong, A.C.L. (1995). Enterotoxin activity of hemolysin BL from *Bacillus cereus*. *Infect. Immun*. **63,** 4423–4428.

Brynestad, S., Synstad, B. and Granum, P.E. (1997). The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiol*. **143,** 2109–2115.

Cornillot, E., Saintjoanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B. and Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Mol. Microbiol*. **15,** 639–647.

D'Aoust, J.-Y., Maurer, J. and Bailey, J.S. (2001). *Salmonella* species. In: *Food Microbiology: Fundamentals and Frontiers*, 2nd ed. (eds. M. Doyle, L. Beuchat and T. Montville), pp. 141–178. ASM press, Washington D.C.

Ehling-Schulz M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M. Märtelbauer, E. and Scherer, S. (2005). Identification and partial characterization of the non-ribosomal peptide synthetase gene esponsible for cereulide production in emetic *Bacillus cereus*. *Appl. Environ. Microbiol*. **71,** 105–113.

Granum, P.E. (1990). *Clostridium perfringens* toxins involved in food poisoning. *Int. J. Food Microbiol*. **10,** 101–112.

Granum, P.E. (1994). *Bacillus cereus* and its toxins. *J. Appl. Bacteriol. Symp. Suppl*. **76,** 61S–66S.

Granum P. E. (2001). *Bacillus cereus*. In: *Food Microbiology: Fundamentals and Frontiers*, 2nd ed. (eds. M. Doyle, L. Beuchat, and T. Montville,.), pp. 373–382. ASM press, Washington D.C.

Granum, P. E. and Brynestad, S. (2002). *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol*. **74,** 195–202.

Granum, P. E. and Harbitz, O. (1985). A circular dichroism study of the enterotoxin from *Clostridium perfringens* type A. *J. Food Biochem*. **9,** 137–146.

Granum, P.E., O'Sullivan, K., Tomas, J.M. and Ørmen, Ø. (1998) Possible virulence factors of *Aeromonas* spp isolated from food and water. *FEMS Immun. Med. Microbiol*. **21,** 131–137.

Granum, P.E. and Richardson, M. (1991). Chymotrypsin treatment increases the activity of *Clostridium perfringens* enterotoxin. *Toxicon* **29,** 898–900.

Granum, P.E., Tomas, J.M. and Alouf, J.E. (1995). A survey of bacterial toxins involved in food poisoning: a suggestion for bacterial food poisoning toxin nomenclature. *Int. J. Food Microbiol*. **28,** 129–144.

Granum, P.E., Whitaker, J.R. and Skjelkvåle, R. (1981). Trypsin activation of enterotoxin from *Clostridium perfringens* type A. *Biochim. Biophys. Acta* **688,** 325–332.

Greco K.M., McDonough M.A. and Butterton J.R. (2004). Variation in the Shiga toxin region of 20th century epidemic and endemic *Shigella dysenteriae* 1 strains. *J. Infect. Dis*. **190,** 330–334.

Hardy S.P., Nakano M. and Iida T. (2004). Single-channel evidence for innate pore formation by *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) in phospholipid bilayers. *FEMS Microbiol. Lett*. **240,** 81–85.

Hammamieh R., Bi S., Mani S., Chakraborty N., Mendis C., Das R. and Jett M. (2003). Genetic variations in peripheral blood mononuclear cells in piglets used as an animal model for staphylococcal enterotoxin exposures. *OMICS*, **7,** 401–409.

Hatheway, C.L. (1990). Toxigenic clostridia. *Clin. Microbiol. Reviews* **3,** 68–98.

Hauschild, A.H.W. (1989). *Clostridium botulinum Foodborne Bacterial Pathogens* (ed. M.P. Doyle), pp. 111–189. Marcel Dekker, New York and Basel.

Jablonski, K.M. and Bohach, G.A. (2001) *Staphylococcus aureus Food Microbiology: Fundamentals and Frontiers*, 2nd ed. (eds. M. Doyle, L. Beuchat, and T. Montville,.), pp. 411–434. ASM press, Washington D.C.

Jackson, S.G., Goodbrand R.B., Ahmed, R. and Kasatiya, S. (1995). *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Lett. Appl. Microbiol*. **21,** 103–105.

Kirov, S.M. (1993). The public health significance of *Aeromonas* ssp in foods. *Int. J. Food Microbiol*. **20,** 179–198.

Kirov, S.M. (2001). *Aeromonas* and *Plesiomonas* species. *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds. M. Doyle, L. Beuchat, and T. Montville,), pp. 301–328, ASM Press, Washington D.C.

Kokai-Kun, J.F. and McClane, B.A. (1997a). Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infect. Immun*. **65,** 1014–1022.

Kokai-Kun, J. F. and McClane, B.A. (1997b). The *Clostridium perfringens* enterotoxin. In *The Clostridia: Molecular Biology and Pathogenesis* (eds. J. Rood, B.A. McClane, J.G. Songer, and R.W. Titball,), 325–357. Academic Press, London.

Kramer, J.M. and Gilbert, R.J. (1989). *Bacillus cereus* and other *Bacillus* species. In: *Foodborne Bacterial Pathogens* (ed. M.P. Doyle,), pp. 21–70. Marcel Dekker, New York and Basel.

Lawson, M.A., Burke, V. and Chang, B.J. (1985). Invasion of HEp-2 cells by fecal isolates of *Aeromonas hydrophila*. *Infect. Immun*. **47,** 1531–1537.

Lund, T. and Granum, P.E. (1996). Characterization of a non-hemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiol. Lett*. **141,** 151–156.

Lund, T. and Granum, P.E. (1997). Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus. Microbiol*. **143,** 3329–3336.

Lund, T., De Buyser, M.L. and Granum, P.E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol. Microbiol*. **38,** 254–261.

Marrack, P. and Kappler, J. (1990). The staphylococcal enterotoxins and their relatives. *Science*. **248,** 705–711.

Lampel, K.A. and Maurelli, A.T. (2001). *Shigella* Species. In: *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds. M. Doyle, L. Beuchat, and T. Montville,), pp. 247–262 ASM Press, Washington D.C.

McClane, B.A. (2001). *Clostridium perfringens*, In: *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds. M. Doyle, L. Beuchat, and T. Montville,), pp. 351–374 ASM Press, Washington D.C.

Miyamoto, K., Chakrabarti, G., Morino, Y., and McClane, B.A. (2002). Organization of the plasmid *cpe* Locus in *Clostridium perfringens* type A isolates. *Infect Immun*. **70,** 4261–4272.

Montecucco, C. and Schiavo, G. (1994). Micoreview: Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol*. **13,** 1–8.

Montoya, M. and Gouaux E. (2003). Beta-barrel membrane protein folding and structure viewed through the lens of alpha-hemolysin. *Biochim. Biophys. Acta*, **1609,** 19–27.

Nachamkin, I. (2001). *Campylobacter jejuni*. In: *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds. M. Doyle, L. Beuchat, and T. Montville,), pp. 179–192. ASM press, Washington D.C.

Nataro, J.P. and Kaper, J.B. (1998). Diarrheagenic *Escherichia coli. Clin. Microbiol. Rev.* **11,** 142–201.

Oliver, J.D. and Kaper, J.B. (2001). *Vibrio* Species. In: *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds Doyle M., Beuchat, L. and Montville, T.), pp. 263–300. ASM Press, Washington D.C.

Park, K.S., Ono, T., Rokuda, M., Jang, M.H., Iida, T. and Honda T. (2004). Cytotoxicity and enterotoxicity of the thermostable direct hemolysin-deletion mutants of *Vibrio parahaemolyticus. Microbiol. Immunol*. **48,** 313–318.

Poly F, Threadgill D, Stintzi A. (2004). Identification of *Campylobacter jejuni* ATCC 43431-specific genes by whole microbial genome comparisons. *J. Bacteriol*. **186,** 4781–4795.

Ray, D.E. (1991). Pesticides derived from plants and other organisms. In: *Handbook of Pesticide Toxicology* (eds. W.J. Hayes, and E.R. Jr. Laws.), pp. 585–636. Academic Press, Inc. New York.

Robins-Browne R.M. (2001). *Yersinia enterocolitica*. In: *Food Microbiology: Fundamentals and Frontiers*, 2. edition (eds. M. Doyle, L. Beuchat, and T. Montville,), pp. 215–246. ASM Press, Washington D.C.

Singh, I., and Virdi, J.S. (2004). Production of Yersinia stable toxin (YST) and distribution of yst genes in biotype 1A strains of *Yersinia enterocolitica*. *J. Med. Microbiol*. **53,** 1065–1068.

Sockett, P. N. and Todd, E.C.D. (2000). The economic costs of foodborne diseases. In: *The Microbiological Safety and Quality of Food* (eds. Barbara Lund, Tony Baird-Parker and Grahame Gould), pp. 1563–1588. Aspen Publishers MD, USA.

Tang, G.Q., Iida, T., Yamamoto, K., and Honda, T. (1995). Ca(2+)-independent cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) on Intestine 407, a cell line derived from human embryonic intestine. *FEMS Microbiol. Lett*. **134,** 233–238.

Wassenaar, T. M. (1997). Toxin production by *Campylobacter* spp. *Clin. Microbiol. Rev*. **10,** 466–76.

Watson, I.M., Robinson, J.O., Burke, V. and Gracey, M. (1985). Invasiveness of *Aeromonas* spp. in relation to biotype, virulence factors, and clinical features. *J. Clin. Microbiol*. **22,** 48–51.

WHO Press Release (1997). hppt://www.who.ch/programmes/fsf

CHAPTER

# 58

# Medical applications of botulinum neurotoxins

*Eric A. Johnson, Gary E. Borodic, and Martin A. Acquadro*

## INTRODUCTION

Since the introduction of botulinum toxin complex (BTX) for the treatment of strabismus in 1980 (Scott, 1989, 2004), the utility of BTX has expanded to many indications across medical specialties. During the past decade, the pharmaceutical utility of BTX has further extended to a variety of disorders that do not have an apparent origin in hyperactive synaptic cholinergic activity. These include various pain disorders, inflammatory conditions, and autonomic syndromes. Important pharmacological attributes of this drug include specificity for cholinergic presynaptic nerve membranes, long duration of action (3–4 months), and the ability to contain the biologic effects within an injected anatomic region. Drawbacks and side effects to BTX use are formation of antibodies in a subset of patients and undesirable spread from the targeted area. Systemic complications have been rare. BTX consists of non-toxic proteins non-covalently linked with the biologically active botulinum neurotoxin (BoNT). Various preparations and immunotypes of BTX have been evaluated in humans with varying results, depending on the toxin serotype, its purity, formulation, potency, and the neurological condition being treated. Advances in understanding of the biochemistry, structure, and cellular biology of BoNTs have provided leads to improve the efficacy of BoNT as a drug and application to new indications. This chapter describes the scientific basis for BTX and BoNT as pharmaceuticals and describes clinical aspects of the toxin.

## NEUROTOXIGENIC CLOSTRIDIA AND BOTULISM

Clostridial neurotoxins (CNTs) are biologically active proteins that are antigenic in animals and can be neutralized with antisera raised against toxoids or against recombinantly expressed toxin fragments (Hatheway, 1988, 1990; Kozaki *et al*., 1989). CNTs, including tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT), are produced by various species of neurotoxigenic clostridia (Hatheway 1989, 1990), and are well-known to cause the spastic and paralytic diseases tetanus and botulism (Bleck 1991; Cherington 1998, 2004). Neurotoxigenic clostridia synthesize TeNT and seven immunologically distinguishable serotypes of BoNTs, designated A through G (Hatheway 1988, 1989). BoNTs can be neutralized by polyvalent antitoxins obtained by immunization of animals with toxoids or recombinant BoNT fragments (Hatheway, 1988; Kozaki *et al*., 1989), or by combinations of monoclonal antibodies generated against BoNTs (Marks, 2004). A specific serotype of BoNT is neutralized by polyvalent antisera to the causative toxin type, but retains toxicity on incubation with antisera raised against a heterologous serotype (Hatheway, 1998).

Botulism is a distinct paralytic disease traditionally caused by the consumption of contaminated food (van Ermengem, 1895). The hallmark of botulism is a descending bilateral weakness and paralysis with an extremely long duration of illness of months to years in severe cases (Cherington, 1988, 2004). Botulism in

humans can weaken or paralyze every skeletal muscle in the body. BoNT inhibition of acetylcholine exocytosis affects parasympathetic and sympathetic systems at neuromuscular synapses. The clinical presentation of severe botulism has been reviewed (Cherington, 1998, 2004). Although the sensory system and CNS mentation are generally unaffected, there have been sporadic reports of sensory abnormalities, which may be associated with an unrelated illness (Cherington, 1998, 2004). Patients with signs of botulism must be monitored for respiratory difficulties. Botulism is quite rare in incidence and may be misdiagnosed as more common paralytic diseases, such as Guillain-Barré syndrome, myasthenia gravis, tick paralysis, diphtheritic neuropathy, Lambert-Eaton syndrome, or certain nervous system infections (Cherington, 1998, 2004). No specific antidote is currently available for preventing botulism or reversing paralysis once receptor binding and internalization ensues. Early administration of antibodies during onset of the disease can decrease the severity and slow its progression, resulting in a shorter hospital stay and more rapid recovery (Centers for Disease Control and Prevention 1998). Complete recovery requires the regeneration and restoration of functional neuromuscular junctions with resumption of neurotransmission.

## BOTULINUM NEUROTOXIN STRUCTURE AND FUNCTION

BoNTs and TeNT are produced as single-chain protein molecules of about 150 kDa that achieve their characteristic high toxicities of $10^7$ to $10^8$ $LD_{50}$s per mg by posttranslational proteolytic cleavage to form a dichain molecule composed of an L chain (about 50 kDa) and a heavy (H) chain (about 100 kDa) linked by a disulfide bond (Habermann and Dreyer, 1986; DasGupta, 1989; Sugiyama, 1980). Most BoNTs produced by strongly proteolytic strains of *C. botulinum* (type A) achieve full activation to the dichain form during culture, while less proteolytic strains of *C. botulinum* (serotypes B–G) produce incompletely nicked BoNTs, and complete activation to the dichain form can be attained in toxin extracts with trypsin or other suitable proteolytic enzymes. Following initial cleavage into the H and L chains, more extensive proteolytic modification may occur, including degradation to inactive forms (Antharavally *et al*., 1998; DasGupta *et al*., 2005). BoNTs can also undergo proteolytic degradation and other modifications during handling and storage, including autocatalytic cleavage and aggregation (Schantz and Johnson, 1992; DasGupta, 2005). Formulations and methods are required to retain the initial high specific toxicity of BoNTs.

BoNT occurs naturally in toxin complexes, commonly referred to as progenitor toxins. In these complexes, BoNT is associated with non-toxic proteins and RNA (Sugiyama, 1980; Sakaguchi, 1983; Schantz and Johnson, 1992; Johnson and Bradshaw, 2001). The full complement of components and structures for these toxin complexes has not been fully elucidated. Progenitor complexes have been referred to as the M complex (about 300 kDa) consisting of BoNT associated with an NTNH protein of 120 to 140 kDa, or as the L complexes (about 450 kDa), in which the M complex associates with hemagglutinin proteins, and the LL (about 900 kDa), where the L complex is thought to form a dimer (Sugiyama, 1980; Sakaguchi, 1983; Schantz and Johnson, 1992). The non-toxic proteins in the complexes have been demonstrated to provide protection during experimental manipulations and during passage through the gastrointestinal tract (Sakaguchi, 1983; Schantz and Johnson, 1992).

The biochemistry, structure, and cell biology of BoNTs have been reviewed (Montecucco and Schiavo, 1995; Schiavo *et al*., 2000; Turton *et al*., 2002; Lalli *et al*., 2003; Simpson, 2004) and is only summarized here as it relates to medical uses of BTX and BoNT. BoNTs and TeNT consist of three basic functional domains: (i) L chain ($L_c$), the catalytic domain that has endopeptidase activity on neuronal substrates; (ii) $H_N$, the translocation domain residing in the N-terminal region of the H chain that enables passage of the $L_c$ from endosomes into the nerve cytosol; and (iii) $H_C$, the receptor-binding domain located in the C-terminal region of the H chain. The gene and amino acid sequences of BoNT and TeNT have been analyzed for a limited number of strains (Niemann, 1991; Lacy and Stevens, 1999; Minton, 1995). The amino acid sequences of all seven BoNTs and TeNT have been deduced from the corresponding genes. The overall amino acid identity is about 40% for the eight CNTs whose genes have been sequenced, and as expected, BoNTs have regions of homology, particularly in the residues defining the catalytic active site, in the translocation domain, and in the two cysteine residues forming the disulfide bond connecting the H chain and the L chain (Niemann, 1991; Lacy and Stevens, 1999). The least degree of homology is in the carboxyl region of the H chain, which is involved in neurospecific binding and probably is the region that determines the serotype. Recent studies have demonstrated that subtypes of BoNTs occur within certain serotypes, in which the 150-kDa proteins differed by 5–20% in amino acid sequence and associated properties such as immunogenicity and neutralization by antitoxins (Dineen *et al*., 2004; Smith

*et al*., 1995; EAJ's laboratory, umpublished data). These findings suggest different evolutionary lineages of CNTs within serotypes leading to distinct BoNT structures, and this finding expands the repertoire of BoNTs available for fundamental studies and evaluation as therapeutics.

Early experiments showed that BoNT and TeNT act at the neuromuscular junction and prevent the release of acetylcholine (Schiavo *et al*., 2000), but the mechanism of action remained enigmatic for many years. During the past decade, it has been revealed that TeNT and BoNTs comprise a unique group of zinc proteases (Montecucco and Schiavo, 1995; Schiavo *et al*., 2000; Breidenbach *et al*., 2004). Their endopeptidase activity was initially suspected by analysis of the gene sequences, in which a highly conserved 20-residue segment contained the zinc-binding motif (His-Glu-Xaa-Xaa-His) characteristic of zinc endopeptidases (Schiavo *et al*., 2000). The identification of the cytosolic substrates for the BoNTs showed that the catalytic $L_c$s specifically cleaved synaptic proteins. BoNTs and TeNT were found to be extraordinarily specific proteases with activity on proteins of the SNARE apparatus (SNAP-25—serotypes A, E, and $C_1$; VAMP/synaptobrevin—serotypes B, $C_1$, D, F, and G; syntaxin—serotype $C_1$) (in Schiavo *et al*., 2000). BoNTs and TeNT cleave the target substrates at different residues, further contributing to their specificity. This high specificity for neuronal substrates is essential for utility of BoNT as a therapeutic, as they do not show general cytotoxicity. Recombinantly expressed $L_c$s also efficiently cleave the substrates *in vitro*, indicating that cytosolic factors are not necessary for activity.

Solving the crystallographic structures of BoNT/A, /B, and /E, as well as BoNT and TeNT domains has led to considerable insight into the cellular biology of these neurotoxins and their utility as pharmaceuticals (Lacy *et al*., 1998; Schiavo *et al*., 2000; Lalli *et al*., 2003; Hanson and Stevens, 2004; Swaminathan *et al*., 2004). The three-dimensional structures of CNTs have been found to be in agreement with the mechanism of intoxication proposed by sequence analysis and biochemical and cell biology experiments (Schiavo *et al*., 2000; Simpson 2004). Lacy *et al*. (1998) demonstrated that BoNT/A holotoxin is composed of domains of different evolutionary origins. The ganglioside-binding C-terminal subdomain has structural homology with proteins known to interact with sugars, such as the $H_C$ fragment of TeNT, serum amyloid P, sialidase, various lectins, and the cryia and insecticidal-$\delta$-endotoxin, which bind glycoproteins and create leakage channels in membranes (Lacy *et al*., 1998; Hanson and Stevens, 2004). Regions of thermolysin and leishmanolysin showed high homology to the catalytic $L_c$ domain. The translocation domain ($H_N$) differed in structure from bacterial pore-forming toxins and showed more resemblance to coiled-coil viral proteins, such as HIV-1, gp41/GCN4, influenza hemagglutinin, and the Moloney murine leukemia virus transmembrane fragment (Lacy *et al*., 1998; Hanson and Stevens, 2004). Interstingly, BoNT consists of a chimera of varied structural motifs that evolved by combination of functional subunits to generate a highly toxic molecule. Recent structural studies for other serotypes of BoNTs, as well as domains of the holotoxin, have shown similarity to BoNT/A and revealed interesting features that provide insight to their high toxicity (Lacy *et al*., 1998; Schiavo *et al*., 2000; Lalli *et al*., 2003; Hanson and Stevens, 2004; Swaminathan *et al*., 2004). Collectively, the biochemical, physiological, and structural studies have led to a pathway for mechanism of action (Montecucco and Schiavo, 1995; Schiavo *et al*., 2000; Simpson, 2004): (a) specific binding of BoNT to the presynaptic membrane of cholinergic neurons; (b) endocytosis of BoNT within vesicles into the nerve cytosol; (c) pH-dependent translocation of the catalytic domain ($L_c$) through the membrane of the endocytotic vesicle into the cytosol; and (d) catalytic cleavage of the specific nerve protein substrates (SNAP-25, VAMP, syntaxin).

The observation that BoNTs are chimeric molecules comprised of distinct protein domains suggests that designed chimeric neurotoxins could be constructed with enhanced or distinct therapeutic utility (Li *et al*., 2001; Goodnough *et al*., 2002; Bade *et al*., 2004). For example, exploitation of the binding and translocation domains to deliver enzymes that kill target neurons could lead to an increased duration of action. Further studies of BoNTs, TeNT, and their domains will undoubtedly lead to increased understanding of the biology of these toxins, as well as providing a strategy for improved vaccines and therapeutic agents.

## PHARMACEUTIC BASIS OF BOTULINUM AND TETANUS TOXINS AS THERAPEUTIC AGENTS

The success of BoNT as a therapeutic derives from certain important attributes of the toxin (Scott, 1989; Johnson, 1999): (a) exceptionally specific binding to the presynaptic membrane of cholinergic terminals; (b) extremely high potency; (c) remarkable specificity for catalytic cleavage of SNARE proteins involved in neurotransmitter trafficking and exocytosis; (d) minimal spread from the injection site; (e) limited and mild adverse effects and; (f) extraordinarily long duration of action.

The exceptional specificity and binding of BoNTs involves a multicomponent binding process to the presynaptic membrane (Montecucco *et al*., 2004). The receptor binding domain of BoNTs lies in the $H_c$ portion of the molecule (Lacy *et al*., 1998; Hanson and Stevens, 2002; Montecucco *et al*., 2004; Swaminathan *et al*., 2004). The presynaptic receptor systems for BoNTs have been postulated to involve an initial capture step mediated by a lipid- or a protein-linked oligosaccharide on the peripheral nerve terminals. This initial capture is followed by interactions with arrays of receptor molecules located in the neuronal membrane (Montecucco *et al*., 2004). These receptor array molecules have not been fully elucidated, but it appears that BoNT/B and BoNT/G interact with synaptotagmin I and II (Dong *et al*., 2003; Rummel *et al*., 2004; Montecucco *et al*., 2004). The possible involvement of dynamic arrays of presynaptic receptors involves more complexity than seen for most hormones and other signaling proteins that bind to receptors. This mechanism also increases the complexity of the ganglioside-protein double-receptor model for TeNT and BoNT that was postulated for many years. The presence of multiple receptor molecules could account for the high affinity of binding, and involvement of different receptors for various serotypes would explain in part the differential specificity for BoNTs. Montecucco *et al*. (2004) have suggested the interesting possibility that binding to the arrays of the presynaptic receptor could trigger a signaling cascade to induce pit formation, capture of BoNTs within a region of limited accessibility (Di Fiore and De Camilli, 2001), including reactivity to antibody molecules and inhibitors. The model proposed (Montecucco *et al*., 2004) is also consistent witht the increase in rate of BoNT internalization on nerve-ending stimulation and small synaptic vesicle (SSV) recycling (Schiavo *et al*., 2000). Since the degree of uptake of BoNTs into nerves is coupled to SSV recycling, the uptake of the toxins, as well as delivery of drugs or inhibitors attached to the receptor-binding domain, would be limited by synaptic neuronal activity. The elucidation of the mechanisms of receptor binding and internalization could lead to improved clinical protocols for BoNT as a therapeutic, as well as novel methods for therapy against botulism intoxication following the internalization process, which are unavailable at this time.

The exceptional toxicity of BoNT is essential for its therapeutic efficacy. BoNT is the most potent toxin known and is biologically active at femtomolar concentrations (Lamanna, 1959; Gill, 1982; Schantz and Johnson, 1992). BoNTs have an estimated lethal intravenous dose of 0.1–1 ng per kg body weight (Lamanna, 1959; Gill, 1982; Schantz and Johnson, 1992; Arnon *et al*., 2001). Experiences with commercial preparations of type A BoNTs in humans as well as non-human primate exposure studies have demonstrated that the intramuscular lethal dose is approximately 39 U/kg body weight for humans (Scott and Suzuki, 1988). Assuming that a mouse $LD_{50}$ of BTX is approximately 30 pg, and monkey susceptibility is equal to that of humans, then the i.m. lethal dose for a 70 kg human would be approximately 0.2 μg (the actual estimated dose is 1–2 μg (Schantz and Johnson, 1992)). When in its native complex form (BTX) (Sugiyama, 1980; Sakaguchi, 1983; Schantz and Johnson, 1992), BoNT is also extremely poisonous by the oral route, with an estimated lethal dose of 70 μg per kg for type A (Arnon *et al*., 2001). Due to the extreme toxicity of CNTs, careful handling and safety measures are extremely important in working with these toxins (Schantz and Johnson, 1992; Centers for Disease Control and Prevention, 1998; Malizio *et al*., 2000).

The specific toxicity of BTX preparations is an important consideration in efficacy and utility of BTX/BoNT as a pharmaceutical. The preparations used in medicine today consist of progenitor toxins, where the active BoNT component exists in large protein complexes. When present in these complexes, the BoNT generally accounts for about 30% of the protein mass, and thus the specific toxicity ($MLD_{50}$ per ng = U) is lower (about 10–40 U per ng) compared to purified neurotoxin (about 80–200 U/ng). It would be expected that the complexes with higher protein mass will have higher immunogenicity than purified BoNT. Furthermore, the biological activities of the non-toxic complexing proteins are not well known, and could have undesirable bioeffects in humans.

A third important attribute of BoNT as a therapeutic is the long duration of action. The longevity of action depends on serotype, and has usually been found to provide durations of paralysis in the order BoNT/A ≥ /C1 > /B> /F> /E in cultured primary neuronal cells (Bergey *et al*., 1987; Keller *et al*., 1999), animal models (Adler *et al*., 2001; Foran *et al*., 2003), and in humans (Eleopra *et al*., 2004). The biological mechanisms for the differences in serotype persistence is currently not understood, but probably involves differences in half-life and degradation of the CNTs in neurons, as well as their effect on neuronal regeneration (Keller and Neale, 2001; Foran *et al*., 2003). Differences in half-life have been inferred from activity measurements of BoNT/A in cultured primary hippocampal cells (Keller *et al*., 1999). The influence of synapse regeneration was inferred by the observation that truncated SNAP-25 modified by BoNT/A (cleavage at $Gln^{197}$-$Arg^{198}$; removal of only eight residues) was shown to remain in the neuronal terminus, while cleavage by BoNT/E

($Arg^{180}$-$Ile^{181}$; greater perturbation by removal of 26 residues) was removed from the neuron with accompanying repletion (Schiavo *et al*., 2000). BoNT/E-truncated SNAP-25 was more rapidly synthesized, presumably leading to more rapid recovery of neuromuscular activity (Eleopra *et al*., 1998). Since SNAP-25 cleaved by BoNT/A was not rapidly removed, it retained (nonfunctional) synaptic structure (Schiavo *et al*., 2000). BoNT/B and /F also have a shorter duration of activity in humans (Ludlow *et al*., 1992; Eleopra *et al*., 2004). The location of the $L_c$s in the nerve cytosol may also contribute to longevity (Dong *et al*., 2004; Fernández-Salas *et al*., 2004), possibly by avoiding proteolysis and removal. Sprouting and formation of new synaptic connections, as well as neuronal plasticity, could also contribute to nerve-muscle connections (Schiavo *et al*., 2000). The elucidation of the mechanisms leading to establishment of productive synaptic connections could provide a basis for BoNT pharmaceuticals with shorter or longer durations of action.

## HISTORICAL DEVELOPMENT OF BOTULINUM TOXIN AS A PHARMACEUTICAL

The background and development of botulinum toxin as a pharmaceutical has been described (Scott, 1989, 2004; Schantz and Johnson, 1992, 1987; Johnson, 1999). The general principle in the development of botulinum toxin as a drug followed the concept of "graded selective denervation" for control of involuntary muscle activity and with retention of voluntary muscle function. The concept of using botulinum toxin as a selective chemodenervating agent was probably considered for many years by neurologists (Scott, 2004). However, Drachman's provocative studies on the effects of botulinum toxin in developing chicken embryos at Johns Hopkins University in Baltimore provided conclusive experimental proof that botulinum toxin A induced selective denervation, muscle weakening, and muscle atrophy (Drachman, 1971). Following injection of enormous quantities (30 μg or approx. 1000 U) of BTX, the thighs and legs of the chicks were markedly shrunken, devoid of muscle tissue, and showed signs of degeneration and fat accumulation. The cardiac muscle of the chicks appeared normal, indicating that botulinum toxin did not have a generalized toxic effect on striated muscle but preferentially poisoned skeletal muscle. In these studies, Drachman obtained "crystalline" BTX preparations from Ed Schantz, who also supplied many researchers with toxin complex for basic and clinical studies (Scott, 2004). Drachman (1971) proposed the characteristics of an "ideal (nerve) blocking agent" in terms of its: (a) **mode of action** in selectively blocking cholinergic transmission (b) **specificity** in blocking only cholinergic transmission; (c) **reversibility** in not permanently impairing function or structure of nerve or muscle; (d) **generality of action** in blocking exocytosis from all motor neuron terminals of striated skeletal muscle; (e) **convenience of use** in requiring simple injection of a soluble toxin preparation into desired muscle regions; (f) **safety** if used with appropriate precautions and proper doses and; (g) **absence of systemic or CNS effects.**

The development of BTX as a therapeutic involved the collaboration of Alan B. Scott and Edward J. Schantz (Johnson, 1999). Scott's initial goal was to provide a pharmacologic alternative to surgery in patients with strabismus (crossed eyes) by injection of extraocular muscles. Compared to other agents tested including ethanol, bungarotoxin, and other substances, accurate injection of graded quantities of crystalline BTX altered ocular alignment. Transient ptosis in the vicinity of the monkeys' eyes cleared within a few days to weeks. Three and one-half months following injection, the EMG recorded from the injected muscle was of normal amplitude and eye movement was also normal. In these experiments, permanent ocular alignment changes after temporary muscle paralysis was the clinical outcome without any need for incision or direct exposure of the muscle. The quantity of toxin needed to induce ocular alignment was approximately 700-fold less than the estimated lethal dose for a 70 kg human (estimated as 1–2 μg; ~1,400 U). Scott predicted that botulinum toxin could be used as a denervating agent in humans for treatment of many conditions characterized by hyperactive neuromuscular activity.

The Food and Drug Administration approved one batch of crystalline botulinum toxin in 1989 produced in 1979 by Schantz at the Food Research Institute, University of Wisconsin - Madison (batch 79–11) for use in the United States for strabismus, blepharospasm, and hemifacial spasm. Schantz (and EAJ) later prepared several batches of crystalline BTX at the University of Wisconsin Food Research Institute using the Schantz strain (Hall A-*hyper*), tested media, fermentation conditions, and purification procedure (Schantz and Johnson, 1992, 1987; Johnson, 1999). According to Scott (personal communication), certain of these batches differed in potency and efficacy on injection into humans. During this period in the mid-late 1980s and extending into the late 1990s, botulinum toxin was increasingly being experimentally used for many other disorders and this single batch of toxin (79–11; ~150 mg) eventually was used for more than 250,000 injections in humans for an increasing number of syndromes.

Scott outlined the following principles and characteristics of botulinum toxin based on his monkey and human studies: (a) it showed no known focal or systemic effects apart from muscle paralysis; (b) it apparently did not elicit antibody production in the small doses that were used; (c) it diffused slowly and to a limited degree out of the injected muscle region into adjacent muscles; (d) the toxin acted for several weeks to months; (e) and the paralytic intensity was strongly correlated with the dose injected. As Scott predicted in 1973 (Scott *et al*., 1973), the utility of botulinum toxin A in medicine has greatly expanded to other neuronal disorders. Undoubtedly, the varieties of disorders treated with botulinum toxin will continue to grow, particularly as we learn more of its basic effects on nerve-muscle physiology.

## EFFECT OF BOTULINUM TOXIN COMPOSITION AND FORMULATION ON PHARMACEUTICAL EFFICACY

Purification of 900-kDa botulinum type A toxin complex (BTX) was refined and perfected in the 1940s–60s at Fort Detrick by Duff, Lamanna, Schantz, and collaborators (see Duff *et al*., 1957; Schantz and Johnson, 1992; 1997). In 1971, Schantz went to the University of Wisconsin, where he developed formulations for stabilization of pg quantities (femtomolar) of type A complex (Schantz and Johnson, 1992). The primary factors investigated were the roles of protein excipients, pH, and sodium chloride. It was decided that flash-dried or freeze-dried preparations were desired to prevent potential contamination and to provide a long shelf-life (Schantz and Johnson, 1992, 1997; Scott, 2004). The primary formulation developed contained human serum albumin (HSA) as a stabilizer (Schantz and Johnson, 1992). Under most drying conditions, the presence of physiological concentrations of NaCl led to BoNT inactivation (Goodnough and Johnson, 1992). Although BoNT was most stable at lower pHs (e.g., 5–6), it was felt that injection of acidic compositions into people could be painful, and thus physiological pH of 7.3 was chosen for the formulation.

Although batch 79–11 (produced in November 1979) was used with excellent results for many years (until 1998), like most proteins it showed degradation over time and specific activity declined to about 4 U per ng (Schantz and Johnson, 1987; Johnson, 1999; Scott, 2004). The decrease in specific toxicity meant that considerable inactive protein was present in the preparation, which probably acted as a toxoid upon injection into humans. This was reflected in the development of antibodies in a proportion of the patients, which then became refractory to further treatment (Hatheway and Dang, 1994; Borodic *et al*., 1996; Atazzi, 2004; Dressler, 2004). In the mid-late 1980s and 1990s, Schantz and I prepared fresh batches with the desired specific toxicity of 30–35 U per ng (Schantz and Johnson, 1992, 1987), which were turned over to Scott and used successfully for treatment of patients (A. B. Scott, personal communication).

During this period in the late 1980s and early 1990s, we were concerned that the complex proteins in the complex form of the toxin could promote immunity in humans based on the early literature that these nontoxic proteins were more immunogenic in animal studies and human trials (Kozaki, *et al*., 1989). Furthermore, experiments at Fort Detrick and elsewhere suggested the non-toxic complex proteins, including the hemagglutinins, could have undesirable biologic properties in humans (M. Crumrine, personal communication). Thus, our laboratory began to investigate the potential of using the pure neurotoxin component (BoNT/A) as the therapeutic agent. One possible concern to the use of BoNT/A instead of BTX was that being a smaller molecule (150 kDa) compared to the crystalline complex (900 kDa), it might diffuse to a greater extent and cause detrimental tissue reactions (Schantz and Johnson, 1987). However, recent experiments in animals (Tang *et al*., 2004; Borodic, unpublished data) and in human clinical trials indicated that tissue spread is not greater than the complex. Methods were developed to formulate and prepare dried preparations of purified BoNT/A, and to provide stability at elevated temperatures. As expected, preliminary experiments indicated that pure BoNT/A was less immunogenic than the large complex (M. Goodnough, thesis, University of Wisconsin). The single protein component in bulk material also enabled more precise analysis of purity and composition, including using SDS-PAGE, and more recently mass spectroscopy, which can be highly discriminating for evaluating the purity of toxin preparations (Barr *et al*., 2005). Thus, current data indicates that pure BoNT/A (and other serotypes) could have advantages in use as a therapeutic for human treatment.

## SAFETY IN WORKING WITH BoNTS AND TeNT

BoNTs and TeNT are extremely toxic molecules and are considered the most potent poisons known. Toxicity of BoNT/A has been estimated as 0.2 ng/kg of body

weight in humans, and the lethal dose is 1 $\mu$g or less. Because the consequences of an accidental intoxication are so severe, safety must be a primary concern of scientists studying these toxins. The CDC recommends biosafety level 3 primary containment and personnel precautions for facilities making large quantities of the CNTs. Selected personnel who work in the laboratory should be immunized with a pentavalent (A–E) toxoid available from the CDC. A biosafety manual should be posted in the laboratory and should contain the proper emergency phone numbers and procedures for emergency response, spill control, and decontamination. When performing steps in which aerosols may be created, special precautions need to be taken. A class II or III biological safety cabinet or respiratory protection should be used. The use of needles and syringes for bioassays requires extreme caution. Beginning in 1997, *C. botulinum* cultures and toxins were included in a group of select agents whose transfer has been controlled by the CDC. To transfer these agents, both the person sending and the person receiving them must be registered with the CDC and exchange the appropriate approval forms (Centers for Disease Control and Prevention, 1998; Malizio *et al.*, 2000).

## CLINICAL ASPECTS

BTXs and BoNTs can be injected transcutaneously into superficial or deep muscles without anesthesia or significant discomfort, through topically anesthetized mucous membranes, or through endoscopes to achieve regional denervating effects. Repeated injections over many years have been used to treat chronic diseases, with few side effects. Few injections over short time periods can be used to treat transient disorders. Placement of agents into appropriate regions is critical to success in treating neuromuscular disease. Diffusion of these agents is a function of dose and composition. Higher dose point injections can traverse fascial barriers causing regional complications within targeted areas, such as dysphagia (difficulty swallowing after anterior neck muscle injections) and diplopia and ptosis (after periorbital muscular injection). Appropriate anatomic placement is also critical to efficacy, as distribution of neuromuscular junctions has been known to vary between different muscle groups. Injection methods that maximize botulinum toxin delivery homogenously throughout the motor axons terminal distribution enhances response (Borodic *et al.*, 1988, 1991, 1994, 1998, 2002). Muscles with parallel fiber orientation tend to have neuromuscular junction distributed to the mid belly, whereas sphincter muscles tend to have diffuse distribution of neuromuscular junctions (Borodic *et al.*, 1988, 1991). For the former muscle type, injections would be most effective if applied to the mid belly, whereas for the sphincter muscle configuration, injections need to be applied within multiple regions. Long muscles such as paraspinal *longissimus dorsi* contain bands of innervation zones corresponding to each spinal nerve root segment (Figure 58.1) (Borodic *et al.*, 1988b, 1990, 1993, 1994). An animal model using multiple muscle biopsies at increasing distance from a point injection has been devised to assess diffusion potential at varying $LD_{50}$ doses (Figure 58.2 and 58.3 B). Such animal models have been used to compare properties of various botulinum toxin preparations for suitability for human use. Use of acetyl cholinesterase staining characteristics and muscle fiber size alterations are significant pathologic markers of botulinum effect, as they are direct structural consequences of neurogenic muscular atrophy (Figures 58.1, 58.2, and 58.3A) (Borodic *et al.*, 1988b, 1990, 1991, 1993, 1994).

The current dose unit is the $LD_{50}$ for the 18–22 g mouse. Such units have proven to be applicable to a particular dose formulation, but cannot be used as a universal standard. Differences in $LD_{50}$ dose requirements in various botulinum toxin preparations have been well documented. Variations in methodology between laboratories can perhaps explain a small degree of deviation in $LD_{50}$ determinations; however, clearly most differences relate to intrinsic pharmacologic properties of a given preparation.

Current research and speculation implicates direct sensory nerve effects from BTX injections ("third bioeffects") (Acquadro and Borodic, 1994; Borodic *et al.*, 2001, 2002a, 2002b; Dressler *et al.*, 2005), which may explain expanded uses of these pharmaceutical agents.

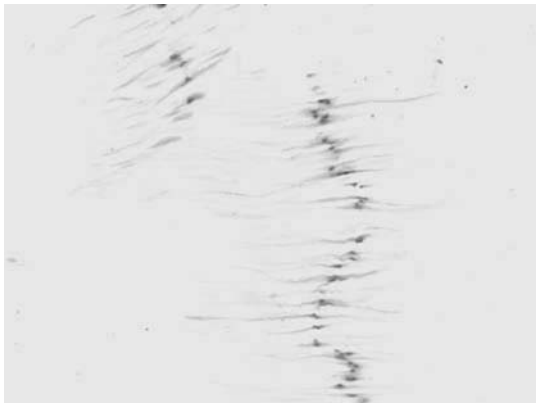


**FIGURE 58.1** Innervation zone of *longissimus dorsi* demonstrated as a band of neuromuscular junctions stained darkly for acetylcholinesterase using histochemistry. Each band of neuromuscular junctions correspond to each spinal segment in the rabbit back.

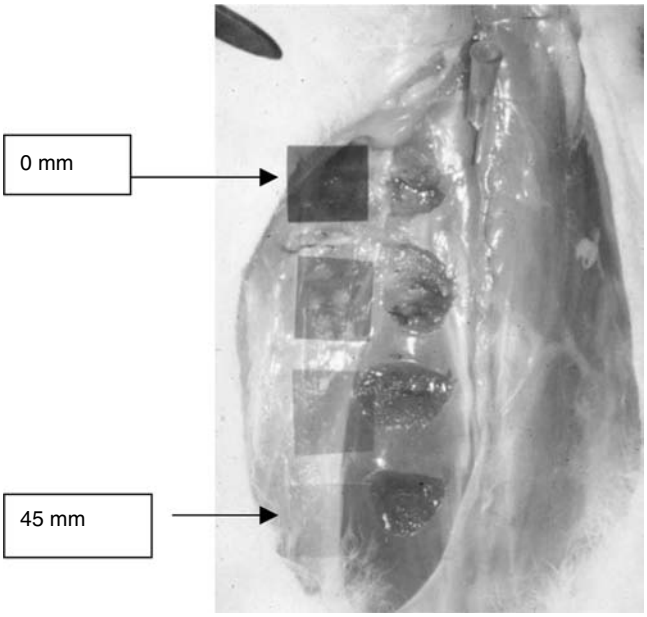



**FIGURE 58.2** Animal model devised to assess diffusion kinetics of botulinum toxin–based pharmaceuticals. Shaded areas corresponded to acetyl cholinesterase staining intensity after point botulinum toxin injections made at the upper segment of the paraspinal muscles (*longissimus dorsi*).

Although sensory nerve effects have been demonstrated in cell culture media (Borodic *et al*., 2002a, 2002b), binding to sensory nerves and definitive sensory nerve changes induced by BoNT remains to be demonstrated. Current research has suggested that secretion and/or intracellular expression of neuropeptides may be influenced by botulinum toxin injections, effecting sensitization and stimulation thresholds. Work in the sensory effects of botulinum toxin is a contemporary topic for basic science investigation. An example of suppression of inflammatory mediators is demonstrated in the skin of a patient treated with involuntary blepharospasm (Figure 58.4). In this example, cholinergic urticaria is suppressed within the denervation-diffusion field around forehead injection sites.

Immunologic resistance has been reported to be clinically significant in the past and continues to be a concern for long-term applications for any preparation (Borodic *et al*., 1996; Dressler, 2004). Upon repeated injections, formation of neutralizing antibodies can occur and highly sensitive methods to test for secondary resistance are still not readily available. Differences in preparation and formulations, such as specific activity, protein load per effective dose cycle, excipients, and immunotype (Borodic and Pearce, 1994; Borodic

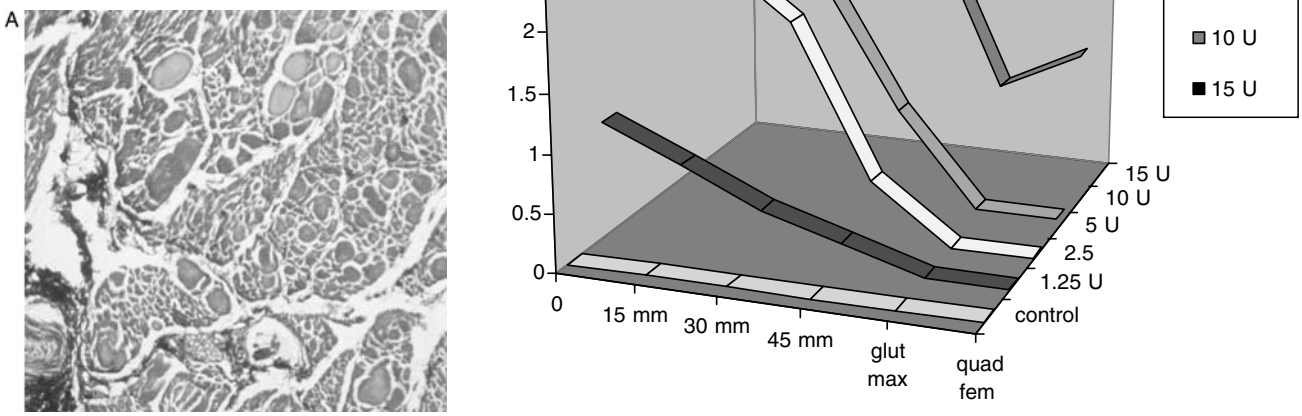



**FIGURE 58.3** (**a**) Muscle fiber size and size variability indicate neurogenic atrophy, a totally reversible phenomenon after botulinum toxin injection. Neurogenic atrophy remains contained to targeted anatomic region based on dose, placement, and composition of materials used. (**b**) Diffusion analysis of a botulinum-based pharmaceutical measured by fiber size variability and spread and intensity of acetyl cholinesterase staining in the rabbit *longissimus dorsi* muscle at increasing distances from injection. The vertical axis represents the degree of denervation using histochemistry and muscle fiber morphometrics; the horizontal axis represents distance from a point injection of varying LD 50 units. Remote location from point injections are gluteus maximus and quadriceps femoris muscles. Note that at 10–15 LD 50 units, the animal's minimum lethal dose, the remote muscles start to become denervated.

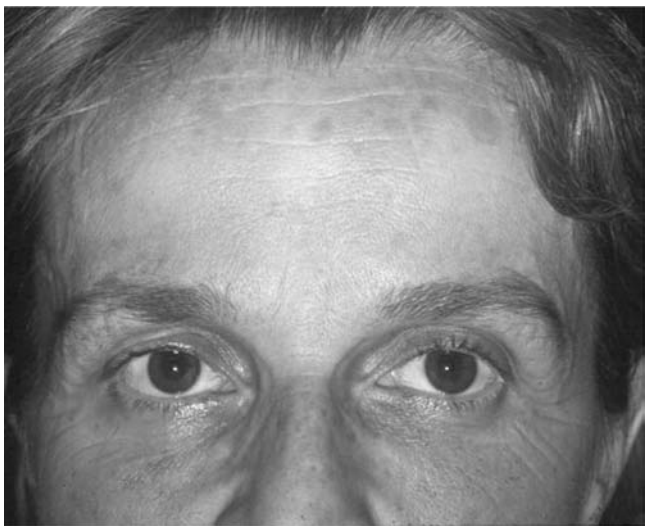


**FIGURE 58.4** "Third bioeffects" are terms related to neither neuromuscular nor autonomic effects of injectable botulinum toxin. Here, a patient demonstrates evidence of such effects on cholinergic urticaria (neurogenic hives) on the forehead. The effect is highlighted by white patches corresponding to the diffusion fields after injected botulinum neurotoxin.

*et al*., 1996) are distinguishing characteristics of available agents. Secondary immune response can render the therapy permanently ineffective. Incidence of antibody formation is an important criterion for assessing suitability of a given preparation for human use. Tachyphylaxis, that is, reduced effectiveness after many injections, occurs, but the role of immunity in this situation is uncertain. Whether this phenomenon is antibody-mediated or caused by another biologic process requires further investigation.

Various methods for measuring botulinum toxin antibodies have been used, including but not limited to neutralization bioassays (mouse neutralization assay), inhibition of Lc catalytic effects on cells in culture, ELISA, immunoprecipitation, and others (Sesardic, 2004; Jankovic *et al*., 2003). The mouse protection assay (MPA) is the standard for measuring antibodies, and the results of this assay have been determined to have consistent predictive value for clinical response (Borodic, 1999; Jankovic, 2004). Other assays, such as mouse hind limb paralysis (Pearce *et al*., 1995), have not been validated against clinical response. ELISA tends to be more sensitive, demonstrating consistently higher positive results compared to mouse neutralization. However, ELISA measures both non-neutralizing and neutralizing antibodies. Other methods for testing for the presence of antibodies are under investigation, using regional denervation models or cell cultures. Ideally, an antibody test should evaluate the entire biological events involved in intoxication, i.e., receptor binding, endocytosis, entry of Lc into the cytosol, and cleavage of neuronal substrates. Several antibody assays have been developed that measure inhibition of the catalytic activity of $L_c$ (Anne *et al*., 2001; Sesardic, 2004). However, these assays only determine one step in BoNT action. Furthermore, it is well-established that the H chain of BoNT contains the majority of epitopes that on immunization lead to neutralizing antibodies (Marks, 2004). Thus, assay systems that involve the $H_c$ and desirably the entire BoNT, such as primary neuronal cell culture methods (Keller *et al*., 1999; Keller *et al.,* Neale, 2004; Sesardic, 2004; Dong *et al*., 2004), the MPA (Pearce *et al*., 1995), and others would provide the optimum test for neutralizing antibodies.

Among current pharmaceutical preparations, more information is needed regarding antigenicity, as patients being treated with these agents often need repeated injections over many years. The current preparation of BOTOX has been reported to have less tendency to cause secondary resistance (Jankovic, 2004), although immunity still can occur, including in low-dose applications such as blepharospasm (Dressler, 2004; Dressler and Saberi, 2005). The original preparation of BOTOX (Allergan) (lot 79–11) was associated with an immunity rate of 10–20% over 3–5 years for large-dose applications (spasmodic torticollis) (BOTOX® package insert). MYOBLOC® (Botulinum immunotype B) has been reported to cause a positive MPA rate of 18% and ELISA positive rate of 50% over 18 months (WE MOVE Internet site).

Duration of action of type B and type F immunotypes has been shown to be significantly shorter than type A at equivalent $LD_{50}$ doses. Some argue that higher doses of type B can result in an almost equivalent duration of action (Lew, 2004). Experience with other immunotypes in human trials has not been reported in significant scale. The mouse $LD_{50}$ unit is not interchangeable and is specific for only one preparation. Generally, the conversion of $LD_{50}$ units between BOTOX/DYSPORT is approximately 4:1, whereas BOTOX /MYOBLOC is 1:100 (Ranoux *et al*., 2002).

The chronology of botulinum toxin injections for the treatment of human disease is presented in Table 58.1. The following represents an overview of indications listed in Table 58.1 with comments on contemporary developments regarding future medicinal utility.

**Ophthalmic Applications (1981)** (Mauriello, 1985, 1987; Elston, 1987; Borodic and Cozzolino, 1989; Snir *et al*., 2003)

In 1980–81, Alan Scott (Smith Kettlewell Eye Institute), with the scientific assistance of Dr Edward Schantz (University of Wisconsin), made a quantum leap in medical discovery by initiating clinical trials studying the application of botulinum toxin for the treatment of deviated ocular alignment in children

**TABLE 58.1**

| Disease | Date therapy initiated | Doses (U) | Case reports/ Open label trials | Randomized controlled studies | Regulatory approval |
|---|---|---|---|---|---|
| Strabismus/related applications | 1981 | 1.25–5 | + | | + |
| Essential blepharospasm | 1982 | 20–80 | + | + | + |
| Hemifacial spasm/facial nerve disorders | 1982 | 15–25 | + | + | + |
| Spamsodic torticollis | 1983 | 100–300 | + | + | + |
| Cerebral palsy | 1985 | | + | | − |
| Spasticity/occupation hand disorders | 1985 | 30–80 | + | + | − |
| Hyperhydrosis | 1988 | | + | + | + |
| Spasmodic dysphonia | 1984 | 2.5–5.0 | + | | − |
| Achalasia/esophageal stricture | 1990 | | + | − | − |
| Sinus secretion/coryza | | | + | | |
| Myofascial pain/headache | 1992 | 50–200 | + | + | − |
| Migraine | 1994 | 20–100 | + | +/− | − |
| Cosmetic (frown lines) | 1993 | 15–50 | + | + | + |
| Tension headache | 1992 | 15–50 | + | +/− | − |
| Post surgical pain | 1994 | 50–100 | + | − | − |
| Temporal mandibular joint syndrome | 1994 | 20–80 | + | + | − |
| Low back pain | 1997 | 100–200 | + | +/− | − |
| Trigeminal neuralgia | 2001 | 30–100 | + | − | − |

+/− indicates conflicting data

(strabismus). Although of substantial historic significance, the initial application for the treatment of strabismus has fallen out of favor as a first line therapy for these conditions due to difficulty with comfortable and effective placement of the pharmaceutical, even with electromyographic injection tip control. Weakness outside the targeted area can lead to vertical eye deviations when horizontal recti muscles are injected. The agents do provide backup options for more difficult surgical situations as an adjunctive measure, such as muscle transposition operations for sixth cranial nerve palsies.

The second and enduring ophthalmic application was neurologic involuntary blepharospasm. This disorder is associated with involuntary facial movements with relentless closure of eyelids, causing not only facial distortion but functional blindness. Botulinum toxin has become the treatment of choice for essential blepharospasm, as well as other related conditions causing involuntary eyelid closure. Blepharospasm was the first movement disease treated with botulinum toxin and the prototype condition from which dosing, principles of injection placement, and diffusion concepts were formulated (Borodic and Townsend, 1988; Borodic *et al*., 1991; Borodic *et al*., 1988, 1993, 1994). Dosing ranges from 15U to 100U of BOTOX® are used for these conditions. Injection placement is important to both efficacy and safety. Multiple injections points are preferred for the treatment within the orbicularis oculli, the major muscle involved with involuntary movement disorders of eyelids. The anatomic distribution of neuromuscular junctions is often diffuse throughout the muscle proper. Multiple injections spread the neurotoxin throughout the muscle, facilitating bringing the neurotoxin to innervation zones. This method has been compared to single-point injections and previously been shown to be superior (Borodic *et al*., 1988, 1991). Furthermore, placement of the material is important in preventing complications associated with this application. Avoiding the midline of the upper lid and medial portion of the lower eyelid help avoid the ptosis and diplopia complications associated with periocular injection. Avoiding placement of the drug away from the lateral canthal tendon avoids toxin diffusion into the lateral orbit structures, including the lateral rectus muscle. Avoiding the medial lower lid mitigates against diplopia from diffusion into the inferior oblique muscle.

The nature of morbidity associated with bilateral blepharospasm is primarily visual loss. Patients with bilateral blepharospasm suffer impairment in routine activities such as driving and reading. Involuntary movement can spread to other regions of the face, causing disfigurement. The extended syndrome has become known as Meige syndrome (previously described as Breugel's syndrome, see Figure 58.5). Prior to the institution of botulinum toxin for these conditions, surgical procedures such as bilateral facial neurectomy and aggressive myectomy surgery would be employed. Despite such procedures, many patients experienced return of involuntary movement with motor nerve regeneration and often became chronically disabled. Botulinum toxin has served to provide the best long-term therapy for this afflictions, as application is sim-

**FIGURE 58.5** Case of *benign essential blepharospasm* with facial dyskinesia (*Meige syndrome*). This was the prototype disease for the application of botulinum toxin for movement disorders, including dystonia, spasticity, and reactive and painful spasms. These patients lose vision and become disabled, based on involuntary eyelid closure.

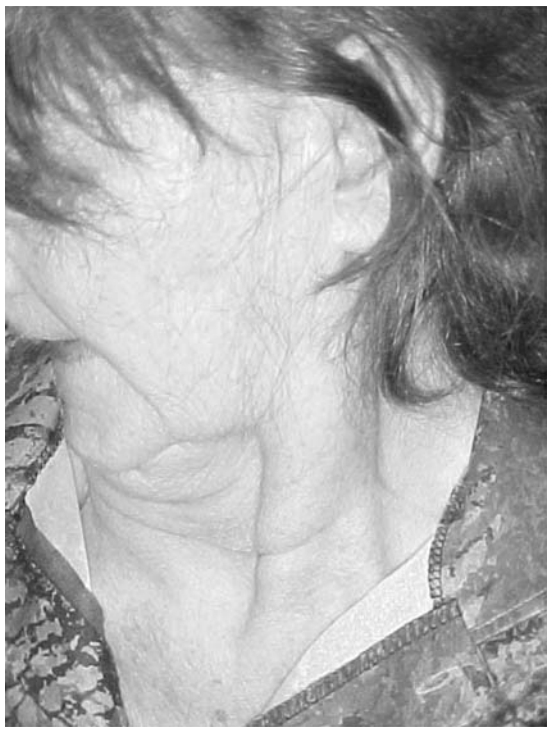


**FIGURE 58.6** Adult onset spasmodic torticollis (cervical dystonia) was the first large-dose indication treated in injectable botulinum toxin-based pharamceuticals. The condition is characterized by pain, posture deformity (tilted, twisted, rotated neck), involuntary movement and tone, decreased range of motion, muscle hypertrophy, and disability. This condition was the prototype for the application of botulinum toxin for human pain disorders and syndromes.

ple, more effective, and safer to repeated surgical interventions.

Other disorders causing involuntary eyelid closure where botulinum toxin treatment has been documented include hemifacial spasm, apraxia of eyelid opening, myokymia, and synkinetic eyelid closure associated with aberrant regeneration of the seventh cranial nerve. Aberrant facial movement remote from periocular location has been effectively treated using botulinum injections throughout other facial regions.

Open label and double-blinded controlled studies have demonstrated efficacy, and regulatory approval had been granted for these indications. These conditions have provided the longest experience for the use of repetitive muscle injections for the treatment of a human affliction.

**Adult Onset Spasmodic Torticollis (Cervical Dystonia) (1983) (Figure 58.6)** (Tsui *et al*., 1986; Brin *et al*., 1987; Stell *et al*., 1988; Gelb *et al*., 1989; Borodic *et al*., 1990, 1991; Jankovic, 2002)

Botulinum has become the therapy of choice for cervical dystonia. This condition is characterized by pain, involuntary movement with distorting and twisting postures of head and neck, decreased range of motion of cervical spines, and hypertrophied sternomastoid muscles (anterior neck muscles). Pain is the most responsive syndrome component for this condition. Injections are usually given in multiple locations, mainly directed at painful regions within the posterior cervical region. Dosing for this indication ranges from 100–300 U with botulinum type A toxin (BOTOX®-OCULINUM) and botulinum toxin immunotype B-(5,000–10,000 U MYOBLOC®). The indication has most commonly been associated with secondary resistance with reported incidence between 4–20% with original botulinum A toxin preparation (79–11). This incidence is probably reduced with more recent preparations of BOTOX® (Jankovic, 2004). Open and double-blinded controlled studies have been reported and U.S. FDA has issued licenses for this indication for both BOTOX and MYOBLOC.

**Spasmodic Dysphonia (1985)** (Benninger *et al*., 2001; Boutsen *et al*., 2002; Pearson *et al*., 2003; Rubin *et al*., 2004)

This condition has been effectively treated with EMG-guided injection into the vocal cords through the cricothyroid membrane. The condition exists in several forms, adductor-abductor types and global muscular involvement, which respond differently to the treatment. The adductor subtype is the most responsive. Doses required are small, ranging from 1.25–5 U BOTOX®. Long-term repeated injections are often necessary. The botulinum injections replaced more destructive neurectomy procedures for this condition.

**Spasticity and Occupational Limb Dystonia (1991)** (Borodic *et al*., 1992; Bakheit *et al*., 2004; Brashear *et al*., 2004; Gordon *et al*., 2004; Swartling *et al*., 2004; Tamura *et al*., 2004)

Spasticity is increased muscle tone with unstable reflex movement caused by injury to the motor cortex and corticospinal tracts within brain and spinal tracts as a result of stroke, cerebral palsy, birth injury (cerebral palsy), head injury, or demyelinating and degenerative nervous system diseases. The clinical morbidity results from upper motor neuron–related weakness and unstable control of movement. Open label trials demonstrating efficacy have been reported for many indications in this category. Placement can require use of EMG stimulating electrodes for correct muscle placement. Doses will vary, depending on size and location of muscle abnormalities. Post-stroke spasticity has been studied with beneficial results demonstrated in double-blinded, placebo-controlled study designs.

**Autonomic Disorders / Hyperhidrosis (1989)** (Hoefflin, 1998; Lowe, 1998; Matarasso; Fagien *et al*., 1999; Naumann and Jost, 2004).

Use of botulinum toxin to reduce sweating and reflex tearing depends on blockage of autonomic cholinergic transmission. Blockage of reflex tearing in patients with aberrant facial nerve regeneration had been initially demonstrated with early experience for periocular injections. Other applications deal with primary hyperhidrosis syndromes, which result in sweating in hands and feet. Doses for these afflictions range from 15 U–100 U (BOTOX)® and regional effects can last for the order of 3–6 months. Double-blinded studies have confirmed efficacy for this indication and U.S. regulatory approval has been issued this past year for this indication.

**Cosmetic Applications (Figure 58.7) (1993)** (Borodic, 1996; Hoefflin 1998; Carruthers *et al*., 2004)

Currently, this is the most common use of botulinum toxin and has affected the general public's perception of BTXs as "serious medicinal agents" because of enormous publicity and promotion given to this treatment. The indication grew out of the observation that periocular lines and frown lines were depressed when the agent was used to treat blepharospasm patients. Masculine-appearing frown lines, forehead lines, and crow's-feet (smile lines), which can give an impression of age, can be effaced by relaxing the resting muscle tone and facial muscle contraction associated with human facial expression. Double-blinded controlled studies were generally simple in intent and design and have demonstrated efficacy for this condition. U.S. FDA approved this indication in 2002. The agents are widely used by healthy individuals for cosmetic purposes.

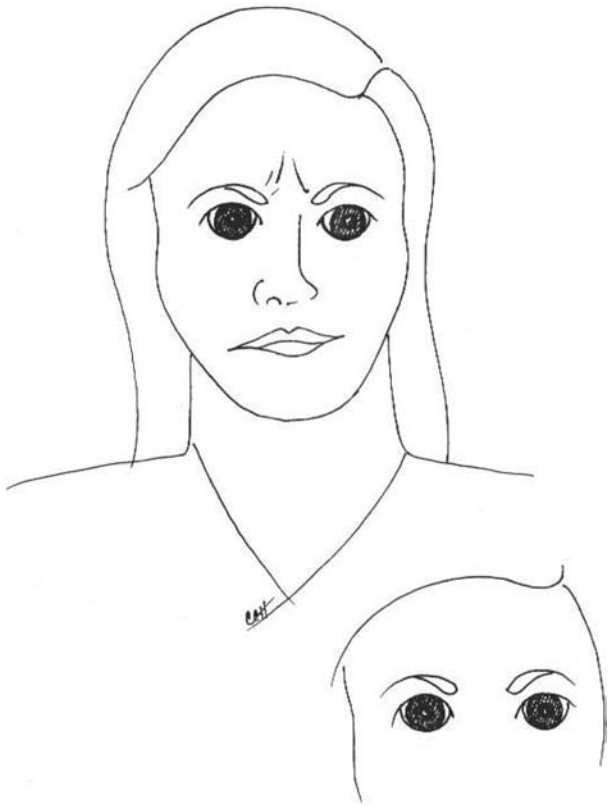


**FIGURE 58.7** The application of botulinum toxin for the reduction of rhytides (wrinkles) has become widespread throughout the world, because of low doses needed to achieve this effect and favorable safety record for this agent. The rhytides targeted include glabellar lines (frown lines between brow, as depicted), crow's-feet (smile lines), forehead creases, as well as neck bands and nasolabial fold flattening.

**Pain (not associated with dystonia)** (Tepper *et al*., 2004; Caputi, 2004; Blumenfeld, 2004; Evers *et al*., 2004; Borodic, 2001; Acquadro, 1993)

The pain component of the spasmodic torticollis-cervical dystonia syndrome was so responsive to botulinum toxin injection that the notion to use botulinum toxin for the treatment of pain emerged (Acquadro 1993). Botulinum was utilized as an agent for the primary treatment of muscle-based pain, without cervical dystonia, that is, myofascial pain. Myofascial pain is a general condition commonly associated with trauma, such as whiplash, surgery, and tension, and essential headaches based on an interaction with the sensory nervous system within various muscular regions. The condition can be non-traumatic in origin. Other overlapping and related medical conditions include fibromyalgia, temporal mandibular joint disease, and stress-related muscle pains. The use of botulinum toxin has rapidly expanded throughout the world for muscle-based applications (Borodic, 2001). Furthermore, observation on possible efficacy in migraine gave further impetus to investigate these indications (Caputi, 2004; Blumenfeld, 2004).

To date, a number of open label studies have suggested efficacy for application of botulinum toxin therapy for headaches (Blumenfeld, 2004). Although an initial industrial sponsored, randomized, double-blinded placebo-controlled study did show efficacy for the treatment of migraine, efforts have failed to reproduce these initial findings and have produced inconsistent results (Evers *et al.*, 2004). Furthermore, double-blinded studies for chronic tension headache have not to date confirmed initial observations (Evers *et al.*, 2004). Many issues relating to confounding data exist in the clinical literature. Endpoints in various studies are different, numbers in controlled studies have been small, no careful subset analysis of response exists, and predictive factors have not been evaluated. The use of retrospective global assessment scales, which have shown efficacy in open label trials and blinded trials, appear to be yielding different outcomes using the diary method of recording symptoms. Headache indications also notoriously are associated with placebo rates as high as 35%, and possibly even higher. An effort to resolve this issue is being conducted with a large multi-institutional double-blind placebo-controlled trial for migraine. Selection criteria for subgroups have also been advocated by the authors at this point in development history. At the time of preparation of this manuscript, more definitive data are expected to be released from large industrial supported, blinded, controlled studies involving the migraine indication.

In summary, pain applications can be a major area for future botulinum toxin use; however, more rigorous randomized controlled clinical studies will be needed to establish absolute scientific proof of efficacy. These studies are difficult because of the inherent high rate of placebo effect associated with migraine/other headache and pain indications and difficulty associated with blinding the treatment groups because of obvious facial and other muscular weakness created by botulinum injections.

### Urologic Applications (Cruz et al., 2004; Smith et al., 2004)

Botulinum toxin was initially applied in the bladder of patients with spinal neurogenic detrusor overactivity and urinary incontinence, or in the urethra in cases of detrusor external sphincter dyssynergia. In addition, the application of botulinum toxin was extended to the treatment of other urological disorders, including non-neurogenic detrusor overactivity, non-relaxing urethral sphincter, and detrusor underactivity. Interesting reports on the injection of botulinum toxin into the prostate of patients with benign prostatic hyperplasia have been reported. Bladder injection of botulinum toxin is not yet an approved treatment for lower urinary tract dysfunction. Nevertheless, available data suggest that in the near future the toxin will become a standard therapeutic option in incontinent patients with neurogenic and non-neurogenic forms of overactive bladder, who do not respond to or do not tolerate anticholinergic medication.

### Other indications

As botulinum toxin has been thought possibly to suppress neurogenic inflammation, other applications through the medical field are possible. Early development case studies are being compiled for interstitial cystitis, dermatosis, internal, and other inflammatory disorders. Cholinergic urticaria has been suppressed over the diffusion field in several patients (Borodic *et al.*, 2001). Experimental evidence in cell sensory cultures suggests botulinum toxin may block neuropeptide secretion, such as substance P, CGRP, and histamine. Despite scattered *in vitro* studies, it still is not clear that botulinum toxin preparations can bind to sensory nerve terminals or the degree that these agents can suppress inflammation.

## CONCLUSION

Botulinum neurotoxin has several desirable properties as a pharamceutical, including exceptional specificity for striated skeletal muscle, localized action and relative absence of systemic effects, remarkably high potency requiring extremely low doses, an uncommonly long duration of action for a single dose, and lack of immunogenicity when manufactured and used properly. It also presently has some disadvantages, such as spread to neighboring muscles, requirement for repeated injections, lack of consistency of biological activity, and antibody formation with improper use. It is likely that many of these disadvantages will be overcome as we better understand the physiology in relation to the human body and new generation botulinum drugs are developed. Despite its current shortcomings, it has benefited thousands of humans and allowed them to live normal and healthy lives.

## REFERENCES

Acquadro, M. and Borodic, G.E. (1994). Treatment of myofascial pain with botulinum toxin. *Anesthesiol.* **80,** 705–6.

Anne, C., Cornille, F., Lenoir, C. and Roques, B.P. (2001). High-throughput fluorogenic assay for determination of botulinum type B protease activity. *Anal. Biochem.* **291,** 253–261.

Anttharavally, B., Tepp, W.H. and DasGupta, B.R. (1998). Status of Cys residues in the covalent structure of botulinum neurotoxin types A, B, and E. *J. Prot. Chem*. **17,** 187–96.

Atazzi, M.Z. (2004). Basic immunological aspects of botulinum toxin therapy. *Movement Disord*. **19** (Suppl. 8), S68–S84.

Arnon S.S., Schechter, R., Inglesby, T.V. and Henderson, D.A. *et al*. (2001). Botulinum toxin as a biological weapon. Medical and public health management. *JAMA* **285,** 1059–70.

Bade, S.A., Rummel, C. Reisinger, Karnath, T., Ahnert-Hilger, G., Bigalke, H. and Binz, T. (2004). Botulinum neurotoxin type D enables cytosolic delivery of enzymatically active cargo proteins to neurones via unfolded translocation intermediates. *J. Neurochem*. **91,** 1461–72.

Bakheit, A.M., Fedorova, N.V., Skoromets, A.A., Timerbaeva, S.L., Bhakta, B.B. and Coxon, L. (2004). The beneficial antispasticity effect of botulinum toxin type A is maintained after repeated treatment cycles. *J. Neurol. Neurosurg Psych*. **75,** 1558–61.

Barr, J., *et al*. (2005). Detection of botulinum neurotoxins by mass spectroscopy, manuscript submitted, and personal communication.

Benninger, M.S., Gardner, G. and Grywalski, C. (2001). Outcomes of botulinum toxin treatment for patients with spasmodic dysphonia. *Arch. Otolaryngol. Head Neck. Surg*. **127,** 1083–5.

Bigalke, H., Wohlfarth, K., Irmer, A. and Dengler, R. (2001). Botulinum A toxin: Dysport improvement of biological availability. *Exp. Neurol*. **168:** 162–170.

Bleck, T.P. (1991). Tetanus: pathophysiology, management, and prophylaxis. *Disease-a-Month* **37,** 547–603.

Blumenfeld, A. (2004). Does single-file botox injection really work for primary headache? *Headache* **44,** 838–9.

Borodic, G.E. (1999). Botulinum toxin issues and applications. *Curr. Opin. Otolaryngol*. **352,** 1832.

Borodic, G.E. and Acquadro, M. (2002). Botulinum toxin for the treatment of chronic facial pain. *Am. J. Pain*. **3,** 21–27.

Borodic, G.E., Acquadro, M. and Johnson, E.A. (2001). Botulinum toxin for the treatment of pain and inflammation. *Expert. Opin. Investig. Drugs* **10,** 1531–44.

Borodic, G.E., Aldersen, K., Pearce, L.B. and Ferrante, R. (1994) Histology and pharmacology of botulinum toxin injections. In: *Botulinum Toxin Therapy* (eds. J. Jankovic and M. Hallet), pp. 119–158 Marcel Dekker, New York.

Borodic, G.E. and Cozzolino, D. (1989). Blepharospasm and its treatment with emphasis on the use of botulinum A toxin. *Plastic Reconst. Surg*. **83,** 546–53.

Borodic, G.E., Cozzolino, D., Weigner, A., Ferrante, R. and Young, R. (1991). Orbicularis oculi innervation zone and implications for botulinum A toxin therapy. *Ophthalmic Plast. Reconstruc. Surg*. **7,** 54–60.

Borodic, G.E., Ferrante, R. and Pearce, L.B. (1993). Review of histologic changes in muscle after clinical botulinum toxin injections and importance of diffusion. In: *Botulinum and Tetanus*, (ed. B.R. DasGupta), pp. 623–645. Plenum Press, New York.

Borodic, G.E., Joseph, M., Fay, L., Cozzolino, D. and Ferrante, R. (1990). Botulinum A toxin for the treatment of spasmodic torticollis, dysphagia, and regional toxin spread. *Head Neck* **12,** 392–8.

Borodic, G.E., Mills, L. and Joseph, M. (1991). Botulinum A toxin for adult onset spasmodic torticollis. *Plastic Reconstruc. Surg*. **87,** 285–9.

Borodic, G.E. and Pearce, L.B. (1994). New concepts in botulinum toxin therapy. *Drug Safety* **11,** 145–52.

Borodic, G.E., Pearce, L.B., Smith, K. and Joseph, M. (1992). Botulinum toxin for spasmodic torticollis, multiple vs. single point injections per muscle. *Head and Neck* **14:**33–7.

Borodic, G.E. and Townsend, D. (1988). Method of injecting botulinum toxin for the treatment of blepharospasm. *Ear Nose Throat J*. **67,** 915.

Borodic, G.E., Weigner, A. and Young, R. (1988). Motor point analysis and botulinum toxin administration. *Ear, Nose, Throat J*. **67,** 915.

Borodic, G.E., Ferrante, R., Wiegner, A.W. and Young, R.R. (1992). Treatment of spasticity with botulinum toxin. *Ann. Neurol*. **31,** 113.

Borodic, G.E., Goodnough, M., Johnson, E.A. and Schantz. (1996). Botulinum toxin, immunologic resistance, and problems with available materials. *Neurol*. **46,** 26–9.

Boyer, A. E., H. Moura, A.R. Woolfitt, S. R. Kalb, L. G. McWilliams, A. Pavlopoulos, J. G. Schmidt, D. L. Ashley, and J. R. Barr. 2005. From the mouse to the mass spectrometer: Detection and differentiation of the endoproteinase activities of botulinum neurotoxins A-G by mass spectrometry. *Anal. Chem*. **77,** 3916–3924.

Boutsen, F., Cannito, M.P., Taylor, M. and Bender, B. (2002). Botox treatment in adductor spasmodic dysphonia: a meta-analysis. *J. Speech Lang. Hear. Res*. **45,** 469–81.

Brashear, A., McAfee, A.L., Kuhn, E.R. and Fyffe, J. (2004). Botulinum toxin type B in upper-limb poststroke spasticity: a double-blind, placebo-controlled trial. *Arch. Phys. Med. Rehabil*. **85,** 705–9.

Brashear, A., Watts, M.W., Narchetti, A., Magar, R., Lau, H. and Wang, L. (2000). Duration of effect of botulinum toxin type A in adult patients with cervical dystonia: a retrospective chart review. *Clin. Ther*. **22,** 1516–24.

Breidenbach, M.A. and Brunger, A.T. (2004). Substrate recognition for botulinum neurotoxin serotype A. *Nature* **432,** 925–29.

Brin, M.F., Fahn, S., Moskowitz, C., Friedman, A., Shale, H.M., Greene, P.E., Blitzer, A., List T., Lange, D. and Lovelace, R.E. *et al*. (1987). Localized injections of botulinum toxin for the treatment of focal dystonia and hemifacial spasm. *Mov/Disord*. **2,** 237–54.

Burgen, A.S.V., Dickens, F. and Zatman, L.F. (1949). The action of botulinum toxin on the neuromuscular junction. *J. Physiol*. **109,** 10–24.

Brin M.F., Hallett, M. and Jankovic, J., eds. (2002). *Scientific and Therapeutic Aspects of Botulinum Toxin*, Lippincott, Williams & Williams, Philadelphia, USA.

Burkhard, F., Chen, F., Kuziemko, G.M. and Stevens, R.C. (1997). Electron density projection map of the botulinum neurotoxin 900-kilodalton complex by electron crystallography. *J. Struct. Biol*. **120,** 78–84.

Caputi, C.A. (2004). Effectiveness of BoNT-A in the treatment of migraine and its ability to repress CGRP release. *Headache* **44,** 837–8.

Carruthers, J., Fagien, S. and Matarasso, S.L. (2004). Botox Consensus Group. Introduction to the consensus recommendations. *Plast. Reconstr. Surg*. **114** (6 Suppl), i–iii.

Centers for Disease Control and Prevention (CDC). (1998). Botulism in the United States, 1899-1996, Handbook for Epidemiologists, Clinicians, and Laboratory Workers. Centers for Disease Control and Prevention, Atlanta, GA.

Chaddock, J.A., Purkiss, J.R., Friis, L.M., Broadbridge, J.D., Duggan, M.J., Fooks, S.J., Shone, C.C., Quinn, C.P., and Foster, K.A. (2000). Inhibition of vesicular secretion in both neuronal and nonneuronal cells by a retrotargeted endopeptidase derivative of *Clostridium botulinum* neurotoxin A. *Infect. Immun*. **68,** 2587–93.

Cherington, C. (1998). Clinical spectrum of botulism. *Muscle Nerve* **21,** 701–10.

Cherington, M. (2004). Botulism: update and review. *Sem. Neurol*. **24,** 155–163.

Comella, C.L., and Pullman, S.L. (2004). Botulinum toxins in neurological disease. *Muscel Nerve* **29,** 628–644.

Cordivari, C., Misra, V.P., Catania, S. and Lees, A.J. (2004). New therapeutic indications for botulinum toxins. *Movement Disord*. **19** (Suppl. 8), S157–S161.

Cruz,, F. and Silva, C. (2004). Botulinum toxin in the management of lower urinary tract dysfunction:contemporary update. *Curr Opin Urol*. **14,** 329–34.

DasGupta, B.R. (1989). The structure of botulinum neurotoxin. In: *Botulinum Neurotoxin and Tetanus Toxin*, ed L.L. Simpson, Academic Press, San Diego, pp. 53–67.

DasGupta, B.R. (2005). Manuscript accepted, personal communication.

Devriese, P.P. (1999). On the discovery of *Clostridium botulinum. J. Hist. Neurosci*. **8,** 43–50.

Dineen, S.S., Bradshaw, M. and Johnson E.A. (2003). Neurotoxin gene clusters in *Clostridium botulinum* type A strains: sequence comparison and evolutionary implications. *Curr. Microbiol*. **46,** 345–52.

Dineen, S.S., Bradshaw, M., Karasek, C. and Johnson, E.A. 2004. Nucleotide sequence and transcriptional analysis of the type A2 neurotoxin gene cluster in *Clostridium botulinum. FEMS Microbiol. Lett*. **235,** 9–16.

Dong, M., Tepp, W.H., Johnson, E.A. and Chapman, E.R. (2004). Using fluorescent sensors to detect botulinum neurotoxin activity *in vitro* and in living cells. *Proc. Natl Acad. Sci. USA* **101,** 14701–6.

Dong, M., Richards, D.A., Goodnough, M.C., Tepp, W.H., Johnson E.A. and Chapman, E.R. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol*. **162,** 1293–1303.

Drachman, D.B. (1971). Botulinum toxin as a tool for research on the nervous system. In: *Neuropoisons: Their Pathophysiological Actions. Vol. 1. Poisons of Animal Origin* (ed. L. L. Simpson), pp. 325–47. Plenum Press, New York -London.

Dressler, D. and Saberi, A.F. (2005). Botulinum toxin: mechanisms of action. *Eur. Neurol*. **53,** 3–9 2.

Dressler, D. (2004). Clinical presentation and management of antibody-induced failure of botulinum toxin therapy. *Movement Disord*. **19** (Suppl. 8), S92–S100.

Dressler, D. and Benecke, R. (2004). Autonomic side effects of botulinum toxin type B therapy. *Adv. Neurol*. **94,** 315–20.

Drobik, C. and Laskawi, R. (1995). Frey's syndrome: treatment with botulinum toxin. *Acta Otolaryngol*. **115,** 459–61.

Duchen, L.W. (1971). An electron microscopic study of the changes induced by botulism toxin in the motor end-plates of slow and fast skeletal muscle fibers of the mouse. *J. Neurol. Sci*. **14,** 47–60.

Duff, J.T. and Wright, G.G. *et al*. (1957). Studies on immunity to toxins of *Clostridium botulinum*. I. A simplified procedure for isolation of type A toxin. *J. Bacteriol*. **73,** 42–7.

Eleopra, R. Tugnoli, V., Quatrale, R., Rossetto, O. and Montecucco, C. (2004). Different types of botulinum toxin in humans. *Movement Disord*. **19** (Suppl. 8), S53–S59.

Elston. J.S. (1987). Long-term results of treatment of idiopathic blepharospasm with botulinum toxin injections: *Br. J. Ophthalmol*. **71,** 664–8.

Ergbuth, F.J. and Naumann, M. (1999). Historical aspects of botulinum toxin: Justinius Kerner (1786–1862) and the "sausage poison." *Neurol*. **53,** 1850–3.

Ergbuth, F.J. (2004). Historical notes on botulism, *Clostridium botulinum*, botulinum toxin, and the idea of therapeutic use of the toxin. *Movement Disord*. **19** (Suppl. 8), S2–S6.

Evers, S., Vollmer-Haase, J., Schwaag. S., Rahmann, A., Husstedt, I.W. and Frese, A. (2004). Botulinum toxin A in the prophylactic treatment of migraine—a randomized, double-blind, placebo-controlled study. *Cephalalgia* **24,** 838–43

Foran, P.G., Mohammed, N., Lisk, G.O., Nagwaney, S., Lawrence, G.W., Johnson, E., Smith, L., Aoki, K.R. and Dolly, J.O. (2003). Evaluation of the therapeutic usefulness of botulinum neurotoxin B, $C_1$, E, and F compared with the long-lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. *J. Biol. Chem*. **278,** 1363–71.

Gelb, D.J., Lowenstein, D.H. and Aminoff, M.J. (1989). Controlled trial of botulinum toxin injections in the treatment of spasmodic torticollis. *Neurology* **39,** 80–4.

Goodnough, M.C. and Johnson, E.A. (1992). Stabilization of botulinum toxin type A during lyophilization. *Appl. Environ. Microbiol*. **58,** 3426–28.

Goodnough, M.C., Oyler, G., Johnson, E.A., Neale, E.A., Keller, J.E., Tepp, W.H., Clark, M., Hartz, S. and Adler, M. (2002). Development of a delivery vehicle for intracellular transport of botulinum neurotoxin antagonists. *FEBS Lett*. **513,** 163–8.

Gordon, M.F., Brashear, A., Elovic, E., Kassicieh, D., Marciniak, C., Liu, J. and Turkel, C (2004). BOTOX® Poststroke Spasticity Study Group. Repeated dosing of botulinum toxin type A for upper limb spasticity following stroke. *Neurology* **63,** 1971–3.

Goschel, H., Wohlfarth, K., Frevert, J., Dengler, R. and Bigalke, H. (1997). Botulinum A toxin therapy: neutralizing and non-neutralizing antibodies—therapeutic consequences. *Exp. Neurol*. **147,** 96–102.

Greene, P.E. (2002). Long-term use of botulinum type F to treat patients resistant to botulinum toxin type A. In: *Scientific and Therapeutic Aspects of Botulinum Toxin* (eds. M.F. Brin, M. Hallett, and J. Jankovic), pp. 451–454. Lippincott, Williams and Wilkins, Philadelphia.

Habermann E. and Dreyer F. (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. *Curr. Top. Microbiol. Immunol*. **129:** 93–179.

Hall, Y. H. J., J. A. Chaddock, H. J. Mouldsdale, E. R. Kirby, F. C. G. Alexander, J. D. Marks, and K. A. Foster. 2004. Novel application of an in vitro technique to the detection and qualification of botulinum neurotoxin antibodies. *J. Immunol. Meth.* **288,** 55–60.

Hanson, M.I. and Stevens, R.C. (2002). Structural view of botulinum neurotoxin in numerous functional states. In: *Scientific and Therapeutic Aspects of Botulinum Toxin* (eds. M.F. Brin, M. Hallett and J. Jankovic), pp. 11–27. Lippincott, Williams and Wilkins, Philadelphia.

Hatheway, C.L. (1988). Botulism. In: *Laboratory Diagnosis of Infectious Diseases: Principles and Practice* (eds. A.Balows A, W.H. Hausler Jr. *et al*.), pp. 111–33. Springer-Verlag, New York.

Hatheway, C.L. (1989). Bacterial sources of clostridial neurotoxins. In: *BotulinumNeurotoxin and Tetanus Toxin* (ed. L.L. Simpson), pp. 3–24. Academic Press, San Diego.

Hatheway, C.L. (1990) Toxigenic clostridia. *Clin. Microbiol. Rev*. **3,** 67–98.

Hatheway, C.L. and Dang, C. (1994). Immunogenicity of the neurotoxins of *C. botulinum*. In: *Therapy with Botulinum Toxin* (eds. J. Jankovic and M. Hallett), pp. 93–107. Marcel Dekker, New York, NY.

Hatheway, C.L. and Johnson, E.A. (1998). *Clostridium*: the spore-bearing anaerobes. In: *Topley and Wilson's Microbiology and Microbial Infections, Ninth Edition, Volume 2: Systematic Bacteriology* (eds. L. Collier, A. Balows and M. Sussman), pp. 731–82. Arnold, London.

Hoefflin, S.M. (1998). Anatomy of the platysma and lip depressor muscles. A simplified mnemonic approach. *Dermatol. Surg*. **24,** 1225–31.

Humeau, Y., Doussau, F., Grant, N.J. and Poulain, B. (2000). How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie* **82,** 427–46.

Jankovic, J. (2004). Treatment of cervical dystonia with botulinum toxin. *Mov. Disord*. **19** (Suppl. 8), S109–15.

Jankovic, J. and Brin, M.F. (1991). Therapeutic uses of botulinum toxin. *N. Engl. J. Med*. **324,** 1186–1204.

Jankovic, J. and Hallett, M., eds. (1994). *Therapy with Botulinum Toxin* Marcel Dekker, Inc., NY.

Johnson, E.A. (1999). Clostridial toxins as therapeutic agents: benefits of nature's most toxic proteins. *Annu. Rev. Microbiol*. **53,** 551–75.

Johnson, E.A. and Bradshaw, M. (2001). *Clostridium botulinum*: A metabolic and cellular perspective. *Toxicon*. **39,** 1703–22.

Johnson, E. A. and Goodnough, M.C. (1998). Botulism. In: *Topley and Wilson's Microbiology and Microbial Infections, Ninth Edition, Volume 3: Bacterial Infections* (eds, L. Collier, A.Balows and M. Sussman), pp. 723–41 Arnold, London.

Keller, J.E., Cai, F. and Neale, E.A. (2004). Uptake of botulinum neurotoxin into cultured neurons. *Biochem*. **43,** 526–32.

Keller, J.E. and Neale, E.A. (2001). The role of synaptic protein SNAP-25 in the potency of botulinum neurotoxin type A. *J. Biol. Chem*. **276,** 13476–82.

Kozaki, S., Kamata, Y., Takahashi, T., Shimizu, T. and Sakaguchi. (1989) Antibodies against botulinum toxin. In: *Botulinum Neurotoxin and Tetanus Toxin* (ed. L.L. Simpson), pp. 301–318. Academic Press, San Diego.

Kreyden, O.P. and Scheidegger, E.P. (2004). Anatomy of the sweat glands, pharmacology of botulinum toxin, and distinctive syndromes associated with hyperhidrosis. *Clin. Dermatol*. **22,** 40–4.

Lacy, D.B. and Tepp, W., *et al*. (1998). Crystal structure of botulinum neurotoxin A and implications for toxicity. *Nat. Struct. Biol*. **5,** 898–902.

Lacy, D.B. and Stevens, R.C. (1999). Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol*. **291.** 1091–1104.

Lalli, G., Bohnert, S., Deinhardt, K., Verastegui, C. and Schiavo, G. The journey of tetanus and botulinum neurotoxins in neurons. *Trends Microbiol*. **11,** 431–7.

Lamanna, C. (1959). The most poisonous poison. *Science* **130,** 763–72.

Li, Y., Foran, P., Lawrence, G., Mohammed, N., Chan-Kwo-Chion, C.K., Lisk, G., Aoki, R. and Dolly, O. (2001). Recombinant forms of tetanus toxin engineered fro examiningand exploiting neuronal trafficking pathways. *J. Biol. Chem*. **276,** 6466–74.

Lowe, N.J. (1998). Botulinum toxin type A for facial rejuvenation United States and United Kingdom Perspectives. *Dermatol. Surg*. **24,** 1216–1218.

Ludlow, C.L., Hallett, M. and Rhew, K. *et al*. (1992). Therapeutic use of type F botulinum toxin. *N. Engl. J. Med*. **326,** 349–59.

Malizio, C.J., Goodnough, M.C. and Johnson, E.A. (2000). Purification of *Clostridium botulinum* type A neurotoxin. *Meth. Molec. Biol*. **145,** 27–39.

Marks, J.D. (2004). Deciphering antibody properties that lead to potent botulinum neurotoxin neutralization. *Movement Disord*. **19** (Suppl. 8), S101–S108.

Mauriello, J.A., Jr. (1985). Blepharospasm, Meige syndrome, and hemifacial spasm: treatment with botulinum toxin. *Neurol*. **35,** 1499–500.

Mauriello, J.A. Jr., Coniaris, H. and Haupt, E.J. (1987). Use of botulinum toxin in the treatment of one hundred patients with facial dyskinesias. *Ophthalmol*. **94,** 976–9.

Minton, N.P. (1995). Molecular genetics of clostridial neurotoxins. In: *Clostridial Neurotoxins* (ed. C. Montecucco), pp. 161–194. Springer-Verlag, Berlin Heidelberg.

Montecucco, C., ed. (1995). *Clostridial Neurotoxins*. Springer, Berlin.

Montecucco, C., Rossetto, O. and Schiavo, G. (2004). Presynaptic receptor arrays for clostridial neurotoxins. *Trends Microbiol*. **12,** 442–446.

Montecucco, C. and Schiavo, G. (1995). Structure and function of tetanus and botulinum neurotoxins. *Quart. Rev. Biophys*. **28,** 423–72.

Moore, P., ed. (1995). *Handbook of Botulinum Toxin Therapy*. Blackwell Science, Oxford.

Naumann, M. and Jost, W. (2004). Botulinum toxin treatment of secretory disorders. *Movement Disord*. **19** (Suppl. 8), S137–S141.

Olney, R.K., Aminoff, M.J., Gelb, D.J. and Lowenstein, D.H. (1998). Neuromuscular effects distant from the site of botulinum neurotoxin injection. *Neurology* **38,** 1780–3.

Park, J.B. and Simpson, L.L. (2003). Inhalational poisoning by botulinum toxin and inhalation vaccination with its havy-chain component. *Infect. Immun*. **71,** 1147–54.

Pearce, L.B., Borodic, G.E., Johnson, E.A., First, E.R. and MacCallum, R. (1995). The median paralysis unit: a more pharmacologically relevant unit of biologic activity for botulinum toxin. *Toxicon* **33,** 217–227.

Pearson, E.J. and Sapienza, C.M. (2003). Historical approaches to the treatment of Adductor-Type Spasmodic Dysphonia (ADSD): review and tutorial. *Neuro. Rehabilitat*. **18,** 325–38.

Pidcock, F.S. (2004). The emerging role of therapeutic botulinum toxin in the treatment of cerebral palsy. *J. Pediatr*. **145**(Suppl 2), S33–5.

Ranoux, D., C. Gury, J. Fondarai, J. L. Mas, and M. Zubar. 2002. Respective potencies of Botox and Dysport: a double blind, randomised, crossover study in cervical dystonia. *Journal of Neurosurgery and Psychiatry*. **72,** 459–462.

Rubin, A.D., Wodchis, W.P., Spak, C., Kileny, P.R. and Hogikyan, N.D. (2004). Longitudinal effects of Botox injections on voice-related quality of life (V-RQOL) for patients with adductory spasmodic dysphonia: part II. *Arch Otolaryngol Head Neck Surg*. **130,** 415–20.

Rummel, A., Karnath, T., Henke, T., Bigalke, H. and Binz, T. (2004). Synaptotagmins I and II act as nerve cell receptors for botulinum toxin G. *J. Biol. Chem*. **279,** 30865–70.

Sakaguchi, G. (1983). *Clostridium botulinum* toxins. *Pharmacol. Ther*. **19,** 165–94.

Schantz, E.J. and Johnson, E.A. 1992. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. *Microbiol. Rev*. **56,** 80–99.

Schiavo, G., Matteoli, M. and Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. *Physiol. Rev*. **80,** 717–66.

Schantz, E.J. and Johnson, E.A. (1997). Botulinum toxin: the story of its development for the treatment of human disease. *Perspect. Biol. Med*. **40,** 317–27.

Scott, A.B. (1989). Clostridial toxins as therapeutic agents. In: *Botulinum Neurotoxin and Tetanus Toxin* (ed. L.L. Simpson), pp. 399–412. Academic Press, San Diego.

Scott, A.B. (2004). Development of botulinum toxin therapy. *Dermatol. Clin*. **k22,** 131–133.

Sesardic, D., Leung, T. and Das, R.G. (2003). Role for standards in assays of botulinum toxins: international collaborative study of three preparations of botulinum type A toxin. *Biologicals* **31,** 265–276.

Sesardic, D., Jones, R.G.A., Leung, T., Alsop, T. and Tierney, R. (2004). Detection of antibodies against botulinum toxins. *Movement Disord*. **19** (Suppl. 8), S85–S91.

Simpson, L.L. (2000). Identification of the characteristics that underlie botulinum toxin potency: implications for designing novel drugs. *Biochimie* **82,** 943–53.

Simpson, L.L. (2004). Identification of the major steps in botulinum toxin action. *Annu. Rev. Pharmacol. Toxicol*. **44,** 167–93.

Simpson, L.L., Madsymowych, A.B. and Kiyatkin, N. (1999). Botulinum toxin as a carrier for oral vaccines. *Cell. Mol. Life Sci*. **56,** 47–61.

Smith, C.P., Radziszewski, P., Borkowski, A., Somogyi, G.T., Boone, T.B. and Chancellor, M.B. (2004). Botulinum toxin A has antinociceptive effects in treating interstitial cystitis. *Urology* **64,** 871–5.

Smith, T.J., Lou, J., Geren, I., Forsyth, C., Tsai, R., Tepp, W.H., Bradshaw, M., Johnson, E.A., Smith, L.A. and Marks, J.D. (2005). Sequence variation within botulinum serotypes impacts antibody binding and neutralization. *Infect. Immun*. (accepted).

Smuts, J.A., Schultz, D. and Barnard, A. (2004). Mechanism of action of botulinum toxin type A in migraine prevention: a pilot study. *Headache* **44,** 801–5.

Snir, M., Weinberger, D., Bourla, D., Kristal-Shalit, O., Dotan, G. and Axer-Siegel, R. (2003). Quantitative changes in botulinum toxin A treatment over time in patients with essential blepharospasm and idiopathic hemifacial spasm. *Am. J. Ophthalmol*. **99,** 105.

Stell, R., Coleman, R., Thompson, P. and Marsden, C.D. (1988). Botulinum toxin treatment of spasmodic torticollis. *BMJ* **297,** 616.

Sugiyama, H. (1980). *Clostridium botulinum* neurotoxin. *Microbiol. Rev*. **44,** 419–448.

Swaminathan, S., Eswaramoorthy, S. and Kumaran, D. (2004). Structure and activity of botulinum neurotoxins. *Movement Disord*. **19** (Suppl. 8), S17–S22.

Swartling, C., Naver, H., Pihl-Lundin, I., Hagforsen, E. and Vahlquist, A. (2004). Sweat gland morphology and periglandular innervation in essential palmar hyperhidrosis before and after treatment with intradermal botulinum toxin. *J. Am. Acad. Dermatol*. **51,** 739–45.

Tamura, B.M., Cuce, L.C., Souza, R.L. and Levites, J. (2004). Plantar hyperhidrosis and pitted keratolysis treated with botulinum toxin injection. *Dermatol Surg*. **30,** 1510–4.

Tepper, S.J., Bigal, M.E., Sheftell, F.D. and Rapoport, A.M. (2004). Botulinum neurotoxin type A in the preventive treatment of refractory headache: a review of 100 consecutive cases. *Headache* **44,** 794–800.

Tsui, J.K., Eisen, A., Stoessl, A.J., Calne, S. and Calne, D.B. (1986). Double-blind study of botulinum toxin inspasmodictorticollis. *Lancet* **8501,** 245–7

Turton, K., Chaddock, J.A. and Acharya, K.R. (2002). Botulinum and tetanus neurotoxins: structure, function, and therapeutic utility. *Trends Biochem. Sci*. **27,** 552–8.

Van Ermengem, E. (1979). Classics in infectious disease. A new anaerobic bacillus and its relation to botulism. *Rev. Infect. Dis*. **1,** 701–19. (Originally published in 1897 as: Ueber einen neuen anaeroben Bacillus und seine Beziehungen zum Botulismus. *Z. Hyg. Infektionskr*. **26,** 1–56.)

Welch, M.J., Purkiss, J.R. and Foster, K.A. (2000). Sensitivity of embryonic rat dorsal root ganglia neurons to *Clostridium botulinum* neurotoxins. *Toxicon* **38,** 245–58.

Whelchel, D.D., Breluner, T.M., Brooks, P.M. and Coffield, J.A. (2004). Molecular targets of botulinum toxin at the mammalian neuromuscular junction. *Movement Disord*. **19** (Suppl. 8), S7–S16.

Williamson, L.C., Halpern, J.L., Montecucco, C., Brown, J.E. and Neale, E.A. (1996). Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J. Biol. Chem*. **271,** 7694–7699.

Wohfarth, K., Kampe, K. and Bigalke, H. (2004). Pharmokinetic properties of different formulations of botulinum neurotoxin type A. *Movement Disord*. **19** (Suppl. 8), S65–S67.

CHAPTER

# 59

# Toxins as tools

*Klaus Aktories*

## INTRODUCTION

Protein toxins are biological instruments, which help bacteria to prevail in a hostile environment defined by the innate or acquired immune system of the host organism. Usually the "classical" toxins are released from the microbes into the environment and, thereafter, act on or in target cells in a manner independently of the presence of the bacteria. To do so, bacteria eveloped fascinating mechanisms to attack the host organism by these microbial instruments, which are outlined in other chapters in detail. These properties of protein toxins are frequently exploited to use them as pharmacological tools to study the cell biology of eukaryotic cells. Among them are toxins, which act on the cell surface to cause pore formations; others act exclusively in the cytosolic fraction of cells and possess elaborate mechanisms to enter cells. Pore formation and uptake mechanisms of toxins are frequently utilized as protein delivery systems to translocate proteins into the cytosol. Many "classical" toxins covalently modify their eukaryotic targets and affect the functions of their targets in a persistent manner, which often results in a permanent inhibition or stimulation of the activities or functions of target proteins, and eventually allows important conclusions about the eukaryotic toxins' targets. Thus, the primary intention of using bacterial protein toxins as tools is to learn more about the biological roles of the eukaryotic targets of microbial toxins. However, very often this inversely results in an increase of our knowledge about the toxins and their structure-function relationships.

### General properties of protein toxins as pharmacological tools

Several properties qualify protein toxins as pharmacological tools (Table 59.1). The toxins are highly specific, extremely potent, and extraordinarily efficient. Moreover, although large in size, many protein toxins are able to translocate into the cytosol without major damage of the cell membrane of the target cells.

The high specificity of toxins is most important for the application of toxins as tools. Evolution of protein toxins resulted in a remarkable specificity for eukaryotic targets. Many toxins are able to differentiate between highly related and structurally similar members of eukaryotic protein families. For example, this is true for signaling proteins of the superfamily of GTP-binding proteins. Certain toxins are able to differentiate between related GTPases of the Rho family or between heterotrimeric GTP-binding proteins. Even more remarkable is the specificity of actin-ADP-ribosylating clostridial toxins to differentiate between α- and β/γ-actin isoforms, which are more than 95% identical in their amino acid sequence (see below, Table 59.2). Noteworthy, many toxins that modify target proteins appear to be more specific in intact cells or tissues than *in vitro* when the substrate specificity is studied under artificial *in vitro* conditions. A typical example for this is cholera toxin, which ADP-ribosylates many proteins in cell lysates (Gill, 1977), whereas no really good evidence exists that the toxins attack other targets than the eukaryotic $G_s$ protein involved in signal transduction via heptahelical receptors (see below). Nevertheless, one has to keep in mind that the

**TABLE 59.1** Advantages of toxins used as tools

| |
|---|
| **1. High specificity** |
| as compared to "synthetic" chemicals used as pharmacological tools action via specific receptors |
| **2. High potency** |
| caused by catalytic mechanisms of many toxins |
| **3. High efficiency** |
| caused by irreversible catalytic mechanism and eukaryotic targets with essential cellular functions |

toxins' specificity may not be "absolute," and it is highly recommended to take the findings obtained with a toxin as *one* important indication that should be corroborated by another toxin or even better by a different method.

High potency of protein toxins means that the toxins act at extremely low concentrations. For example, botulinum neurotoxins are the most potent biological agents ever known, with a minimal lethal dose in mice in the range of 10 picogram (Payling-Wright, 1955). These high potencies of toxins are achieved by at least two properties. First, many toxins are enzymes and act in a catalytic manner. They are, for example, proteases, ADP-ribosyltransferases, glucosyltransferases, N-glycosidases, or deamidases. Therefore, only few molecules may be necessary to modify target proteins by a catalytic reaction. Even more, it has been estimated that only one molecule of the ADP-ribosyltransferase diphtheria toxin, which inhibits protein synthesis by modification of elongation factor 2, is sufficient to intoxicate a target cell (Yamaizumi *et al*., 1978). Second, by high-affinity interaction with specific cell surface receptors, the toxins may target preferentially cells that are particularly sensitive towards the toxins' actions.

**TABLE 59.2** Toxins most often used as tools

| | |
|---|---|
| **I.** | **Pore-forming toxins** |
| | Streptolysin O |
| | Staphylococcus aureus α-toxin |
| | Tetanolysin |
| **II.** | **Toxins targeting heterotrimeric G proteins** |
| | Cholera toxin for $G_s$ |
| | Pertussis toxin for $G_{i,o}$ |
| | Pasteurella multocida toxin for $G_q$ |
| **III.** | **Toxins targeting low molecular mass GTPases** |
| | C3 exoenzymes for inactivation RhoA, B, and C |
| | *Clostridium difficile* toxin A and B for inactivation of Rho family GTPases |
| | *Clostridium difficile* toxin B variant 1470 for inactivation of Rac but not RhoA |
| | *Clostridium sordellii* lethal toxin for inactivation of Rac but not RhoA |
| | *Escherichia coli* CNF for activation of Rho GTPases |
| **IV.** | **Toxin targeting actin** |
| | *Clostridium botulinum* C2 toxin (non-α-actin isoform) |
| | *Clostridium perfringens* iota toxin (all actin isoforms) |
| **V.** | **Neurotoxins targeting synaptic proteins** |
| | *Clostridium botulinum* neurotoxins |
| | BoNT/A for SNAP25 |
| | BoNT/B, TeNT for synaptobrevin |
| | BoNT/C for syntaxin |
| **VI.** | **Toxin-based cell delivery systems** |
| | Diphtheria toxin, *Pseudomonas* exotoxin A |
| | *Clostridium botulinum* C2 toxin |
| | Anthrax toxin |

The high efficiency of bacterial toxins is caused by reasons already mentioned. Of course, the catalytic activity, which is the basis for the mode of actions of many toxins, is particularly important. However, similarly important for high efficiency is the toxins' choice of eukaryotic substrates. Generally, the toxins attack functionally important eukaryotic substrates, which play pivotal roles in the biology of the target cell. These toxins' targets are often crucial switch proteins of cellular signaling like heterotrimeric G proteins. For example, this is true for the activation of the $G_s$ proteins, which are ADP-ribosylated by cholera toxin, and also for the Gi proteins, which are inhibited by pertussis toxin-catalyzed ADP-ribosylation. Perhaps an even more impressive example is the targeting of Rho GTPases by various bacterial protein toxins, including Rho-inhibiting C3 ADP-ribosyltransferases and clostridial glucosylating toxins (see below). These signaling proteins are not only inhibited but also activated by toxins like the deamidating cytotoxic necrotizing factors (CNF 1 and 2) and the transglutaminating dermonecrotic toxin DNT.

It is important to mention that not only the "classical" protein toxins, which may be defined by their ability to act on target cells independently of the presence of the producing bacteria, are employed as pharmacological tools. Recently, bacterial effector proteins, whose cell entry depends on the presence of the bacteria and which are microinjected by type-III secretion system (TTSS) into the target cells, also are used as toxins. In this case, the complete bacteria must be used, which has many drawbacks, e.g., the specificity of the action.

## PORE-FORMING TOXINS AS TOOLS

Different types of bacterial protein toxins are known that affect target cells by formation of pores, thereby causing imbalance of cellular electrolytes, depletion of nucleotides, or other essential low molecular mass cellular factors. These toxins are widely used as pharmacological tools to permeabilize eukaryotic cells with the intention to change the cytosolic content in a well-defined manner; for example, to introduce small molecules, which are membrane impermeable (e.g.,

nucleotides and ions). The pore-forming toxins are used to introduce peptides or proteins, such as antibodies, into target cells

Streptolysin O from *Streptococcus pyogenes* is probably the best established pharmacological tool in this group (Ahnert-Hilger *et al*., 2000). However, many other pore-forming toxins are known that are used as cell biological tools, including tetanolysin, *Escherichia coli* hemolysin, and *Staphylococcus aureus* α-toxin (van der Goot, 2001). Interestingly, usage of pore-forming toxins as delivery devices appears not to be an artificial application of these toxins. It was reported recently that streptolysin O functions as a delivery system to allow other bacterial proteins that lack a cell transportation device to enter host cells. Therefore, usage of pore-forming toxins as a pharmacological tool to deliver membrane impermeable factors into the cytosol might mimic their biological function (Madden *et al*., 2001).

Streptolysin O is an approximately 60-kDa protein, which belongs to the family of cholesterol-dependent cytolysins, indicating that its action depends absolutely on the presence of cholesterol (Bhakdi *et al*., 1997). Streptolysin O and related toxins (e.g., perfringolysin O) interact with membrane cholesterol and homo-oligomerizes to form a prepore. Then the toxin oligomers convert to a pore-forming complex by membrane insertion of two transmembrane β-hairpins per monomer and form large aqueous β-barrel pores with an internal diameter of up to 30 nm. About 50 monomers take part in oligomerization and pore formation. The toxin does not bind to membranes, which do not contain cholesterol. Moreover, the number of binding sites and the binding affinity appear to depend on the amount of cholesterol in the membrane (Palmer, 2001).

Because the pores of streptolysin O are very large, they are permeable for large molecules (over 100 kDa). Probably, this is the reason why streptolysin O also interacts with internal membranes. When the toxin is applied as a tool, the extent of cell damage and the alteration of intracellular membranes are reduced by dissociation of the binding event from the pore-forming process by a temperature shift. First, streptolysin O binding is carried out at low temperature (0°C). Then surplus streptolysin O is removed by washing. The permeabilization is initiated by increasing the incubation temperature to 37°C. Usually, the permeabilization by streptolysin O is lethal for the cell. However, recently it was shown that a reversible membrane permeabilization is possible with streptolysin O. This depends on calcium-calmodulin and intact microtubules and allows the transport of up to 100-kDa proteins into the cytosol (Walev *et al*., 2001).

Pore-forming toxins have been widely used in studies on exocytosis from various secretory cells, on calcium regulation, on signal-contraction coupling in smooth muscle cells, and on intracellular membrane trafficking (Ahnert-Hilger *et al*., 2000). Researchers using pore-forming toxins must consider that these toxins induce a wide spectrum of biological effects, many of which are explained by changes in cellular ion fluxes and appear to be triggered by monovalent ion fluxes and by $Ca^{2+}$ influx (e.g., secretion, generation of lipid mediators, and cytoskeletal redistribution). However, some of the effects of pore-forming toxins cannot be explained in this way. Examples of the latter effects are activation of G-protein signaling and proteolytic shedding of membrane proteins. All these effects must be considered when interpreting data obtained with pore-forming toxins as tools.

Other members of this large toxin family are perfringolysin O from *C. perfringens*, and tetanolysin from *C. tetani*.

*S. aureus* alpha-toxin, which is also frequently used as a pharmacological tool, is a hydrophilic polypeptide of 293 amino acids, whose crystal structure was analyzed recently (Song *et al*., 1996). The model derived from the crystallographic data shows a mushroom-shaped, homo-oligomeric heptamer with a transmembrane domain composed of a 14-strand antiparallel β-barrel with a pore diameter of 2.6 nm. The pore formation caused by alpha-toxin is limited to the cell membrane because the toxin monomers (about 33 kDa) are not able to pass through the pore.

### Pore-forming toxins used as pesticides

It should be mentioned that some pore-forming toxins have an enormous commercial importance as pesticides against insects. For this purpose especially, members of the large family of pore-forming toxins (Cry families) from *Bacillus thuringiensis* are used. These toxins kill insect larvae, but apparently are not harmful to mammals, and spraying plants with spores of *B. thuringiensis* seems to be environmentally safe. Importantly, the various toxins are specific for certain insects only. Recently, the genes for toxins were introduced into many crop plants in an effort to protect them from insect attack (Schnepf *et al*., 1998; Aronson and Shai, 2001).

## PROTEIN TOXINS TO STUDY GTP-BINDING PROTEINS

Several ADP-ribosylating toxins target GTP-binding proteins, which belong to related GTPase families.

While diphtheria toxin and *Pseudomonas* exotoxin A modify the eukaryotic GTP-binding protein elongation factor 2, cholera toxin, the related heat-labile toxins from *E. coli*, and pertussis toxin modify the α-subunits of heterotrimeric G-proteins from Gs and Gi, respectively. In addition, several low molecular mass GTPases of the Ras superfamily are targets of ADP-ribosylating toxins, including C3-like transferases from *C. botulinum*, *C. limosum*, and *S. aureus*.

## Cholera toxin and pertussis toxin

Cholera toxin and pertussis toxin have been extremely valuable tools in studies on signal pathways involving heterotrimeric G proteins (Figure 59.1).

Cholera toxin is produced by *Vibrio cholerae* and the cause of the watery diarrhea of cholera. The ~85-kDa toxin is composed of an enzyme subunit of about 28 kDa (A-subunit) and a binding component consisting of five identical B subunits of about 11.6 kDa. The enzyme part consists of a ~21-kDa A1 and a ~6-kDa A2 subunit. A1 and A2 are linked by a disulfide bridge. A1 is the ADP-ribosyltransferase, whereas A2 links the enzyme to the B component. The B subunit binds to the ganglioside receptor GMI, which leads to internalization of the toxin and retrograde transport to the ER and translocation of the enzyme subunit via the sec61p into the cytosol (Kaper *et al*., 1995). The ADP-ribosyltransferase targets the α-subunit of heterotrimeric Gs proteins. It ADP-ribosylates arginine 201 of $G_{s\alpha}$ or equivalent arginine residues in splice variants of the α subunit of the $G_s$ protein. The modification of $G_{s\alpha}$ blocks its intrinsic GTPase activity, prevents the endogenous switch-off mechanism, and renders the α subunit persistently active (Cassel and Pfeuffer, 1978). In the intestinal mucosa, persistently active $G_{s\alpha}$ stimulates adenylyl cyclase, increases intracellular cyclic AMP levels, and activates cAMP-dependent kinase A (PKA), which phosphorylates numerous proteins. It is suggested that phosphorylation of $Cl^-$ channels (e.g., CFTR) finally results in electrolyte and fluid misbalance typical for cholera. *In vitro*, cholera toxin also ADP-ribosylates other G proteins such as transducin and, under specific conditions, even $G_i$ (Gierschik *et al*., 1989). In addition, potential *in vitro* substrates include many cell proteins with a suitable arginine residue. Also, small arginine derivatives like agmatine are *in vitro* modified. Finally, the toxin itself is modified by an auto-ADP-ribosylation reaction. Thus, in *in vitro* experiments, the specificity of CT is not very impressive. By contrast, in intact cells, $G_{s\alpha}$ seems to be the preferred substrate of the toxin (Van den Akker *et al*., 2000). Like many other ADP-ribosyltransferases, CT has NAD glycohydrolase activity. It splits NAD into ADP-ribose and nicotinamide in the absence of a protein

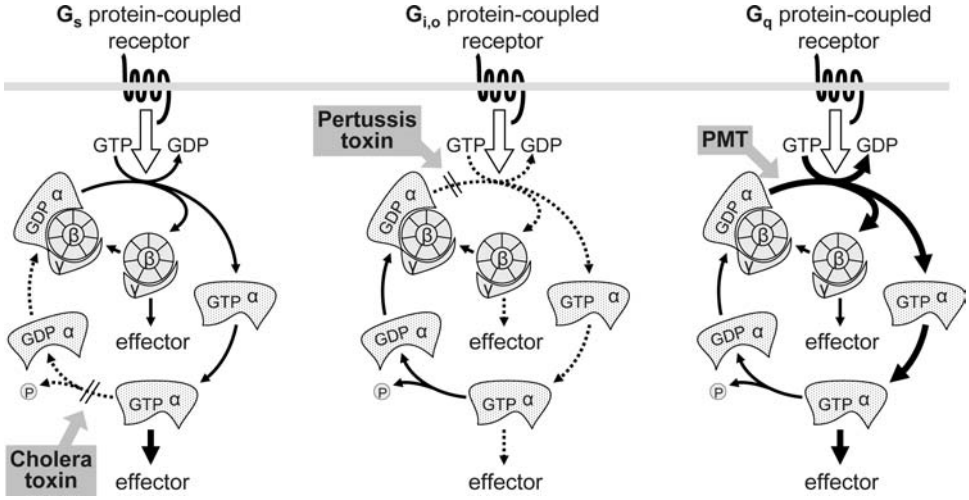



**FIGURE 59.1** Mode of action of toxins affecting heterotrimeric G-proteins.
Left panel: Cholera toxin ADP-ribosylates the α-subunits of Gs proteins. This blocks the GTPase activity and inhibits the switch-off mechanism of the Gs protein.
Middle panel: Pertussis toxin ADP-ribosylates $G_{i,o}$ proteins. Thereby, the interaction of the G protein with the receptor is blocked. The G protein cannot be activated by the receptor and remains in the inactive state.
Right panel: *Pasteurella multocida* toxin (PMT) activates $G_q$ proteins. Although the precise mechanism is not known at present, it is suggested that the activation step is facilitated by the toxin. This causes enhanced activation of $G_q$ signaling.

substrate. NAD glycohydrolase activity is low as compared with the ADP-ribosyltransferase activity. ADP-ribosylation by CT is stimulated by the presence of ADP-ribosylation factors, which are members of a subfamily of small GTPases (ARF subfamily of GTPases). In addition to application of CT as a tool to manipulate signal pathways via $G_s$ protein or to label $G_{s\alpha}$ protein, the toxin is frequently used as an adjuvant to stimulate mucosal immune responses (Plant and Williams, 2004). Moreover, CT is successfully used as a tracer for retrograde transport in neuronal cells (Hayakawa *et al*., 2004).

Pertussis toxin (PT) is a major exotoxin of *Bordetella pertussis*, the causative pathogen of whooping cough. PT is a hexameric toxin of 105 kDa, consisting of the enzyme subunit S1 (about 26 kDa, A subunit) and the binding pentamer (B subunit) formed of S2 (about 22 kDa), S3 (about 22 kDa), two S4 subunits (about 12 kDa), and S5 (about 12 kDa). PT ADP-ribosylates the $G_\alpha$ subunits of the heterotrimeric $G_{i/o}$ proteins (e.g., $G_{i1\text{-}3}$, $G_{o1,2}$, and $G_t$) (Figure 59.1). The toxin selectively ADP-ribosylates a cysteine residue located at the C-terminus (four amino acid residues before the carboxy-terminal end) of the Gα proteins. In the 1980s, PT was most important for identification of $G_i$ as inhibitory G proteins besides the stimulatory $G_s$ protein. A consequence of PT-catalyzed ADP-ribosylation is uncoupling of G proteins from their membrane receptors with subsequent blockade of the G-protein-transduced signal pathways. Importantly, G proteins of the $G_s$, $G_{q/11}$, $G_{12/13}$, and $G_z$ subfamilies and a splice variant of $G_{i2}$ [$G_{i2}$(L)] are not modified by pertussis toxin. Therefore, the toxin is a well-established tool to test whether a signaling pathway involves "PT-sensitive" G proteins or not (Nürnberg, 1997).

## *Pasteurella multocida* toxin

Recently, *Pasteurella multocida* toxin was employed for studies involving $G_q$ proteins. PMT is a ~ that 150-kDa protein that causes atrophic rhinitis and turbinate bone atrophy in pigs (Lax *et al*., 2004; Wilson and Ho, 2004). It is an extremely potent mitogen. One prominent toxin effect is stimulation of phospholipase Cβ (Murphy and Rozengurt, 1992). So far, the precise molecular mechanism of toxin is not known. However, recent studies indicate that PMT activates $G_{\alpha q}$ proteins (Wilson *et al*., 1997) (Figure 59.1). Therefore, PMT is applied in signal transduction studies to investigate the role of $G_q$. Interestingly, PMT is able to differentiate between $G_q$ and $G_{11}$. Gq stimulates $G\alpha_q$, but not the highly related $G\alpha_{11}$. In addition PMT interferes with Rho signal pathways (see below) (Zywietz *et al*., 2001). Rho proteins are most likely activated by $G_q$, but also by the $G_{12/13}$ subfamily proteins. Therefore, it might be that the effect of PMT is not as specific as previously suggested and also $G_{12/13}$ proteins are activated by the toxin.

## Studying small GTPases with bacterial protein toxins

Various low-molecular-mass GTP-binding proteins are targets of bacterial protein toxins, which cause inhibition or stimulation of these switch proteins. Well-studied examples of the toxins are the ADP-ribosylating C3-like transferases, the clostridial glucosylating toxins, and deamidating and/or transglutaminating toxins. All these toxins were frequently used as cell biological tools and were of major importance in elucidating the functional role of the small GTPases.

Small GTPases are usually grouped in at least five families, including the Ras, Rab, Arf, Ran, and Rho subfamilies, which have about 30% sequence identity. Members within each GTPase subfamily are at least 50% identical in their amino acid sequence. Similar to heterotrimeric G proteins, the low molecular mass or "small" GTP-binding proteins are regulated by a GTPase cycle. They are inactive in their GDP form and active after GDP-GTP exchange. Hydrolysis of bound GTP terminates the active state of the GTPases (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000). Especially Rho subfamily GTPases are targets for bacterial protein toxins. This family comprises about 20 GTPases (e.g., RhoA, RhoB, RhoC, RhoD, RhoE, RhoG, Rac1, Rac2, Rac3, Cdc42Hs, G25K, and RnD/RhoE). At least three groups of regulatory proteins control the function and activity state of Rho proteins. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by facilitating the GDP-GTP exchange. GTPase-activating proteins (GAPs) stimulate and catalyze the GTP hydrolysis, thereby inactivating the GTPases and, finally, guanine nucleotide dissociation inhibitors (GDIs). The latter regulators block nucleotide exchange, extract Rho proteins from membranes, and keep them in the cytosol. Most likely, they are involved in presentation of the GTPases to effectors or other regulators.

Rho GTPases (Rho, Rac, and Cdc42) are master regulators of the actin cytoskeleton. They are crucially involved in adhesion and migration of cells. They play a pivotal role in endocytosis, secretion, and phagocytosis. Moreover, Rho GTPases act as molecular switches in numerous vital cellular processes, including cell polarity, cell cycle progression, transcriptional regulation, transformation, and apoptosis (Nobes and Hall, 1995).

# INACTIVATION OF RHO GTPases BY BACTERIAL PROTEIN TOXINS

## C3-like ADP-ribosyltransferases

C3-like ADP-ribosyltransferases are basic proteins (IP > 9) with molecular masses of about 25 kDa, having a sequence identity of 30 to 70%. Seven isoforms produced by *Clostridium botulinum, Clostridium limosum, Bacillus cereus*, and *Staphylococcus aureus* have been described. C3 transferases ADP-ribosylate RhoA, RhoB, and RhoC at asparagine 41, thereby blocking the biological activity of Rho (Figure 59.2). The underlying mechanism is most likely inhibition of activation of Rho GTPases by GEFs, release from membranes into the cytosol, and formation of a tight inactive Rho-GDI complex after ADP-ribosylation (Sehr *et al*., 1998; Genth *et al*., 2003). Although asparagine 41 is located in the so-called effector region of the Rho protein, ADP-ribosylated Rho is still able to bind to its effectors' protein kinase N and Rho-kinase. C3 modifies Rac by up to 5% *in vitro*. However, all experimental evidence indicates that in intact cells, C3-like transferases ADP-ribosylate RhoA, RhoB, and RhoC, but not Rac and Cdc42. This is most important because C3 has been widely used to study the cell biological role of Rho. There is an exception: C3stau (also called EDIN) additionally ADP-ribosylates RhoE/(Rnd3) (Wilde *et al*., 2001). Recent studies suggest that some C3 isoforms possess additional properties, that might be of relevance for using the toxins as tools. For example, it was shown that C3 from *C. botulinum* and *C. limosum* interacts with high affinity (about 15 nM) with the small GTPase Ral without ADP-ribosylating the protein. This interaction, however, blocks the ADP-ribosylation of Rho by C3 (Wilde *et al*., 2002). Because Ral functions are also altered by C3 binding (at least *in vitro* activation of phospholipase D by Ral is blocked by C3), it is feasible that high concentrations of C3, which are used for microinjection studies, affect Ral signaling.

C3 transferases lack any specific binding and translocation subunit and their cell accessibility is rather poor. This is probably the reason why C3 shows low toxicity compared with other protein toxins. For example, the intraperitoneal injection of 100 µg of C3 into mice has no obvious consequences. To circumvent the problem of poor cell accessibility, several approaches are possible. First, various techniques to permeabilize cells can be applied to allow C3 uptake. Osmotic shock, electroporation, and pore-forming toxins like streptolysin O were used for this purpose. Many studies applied microinjection techniques (Paterson *et al*., 1990). Other approaches are based on chimeric toxins. A fusion toxin, consisting of C3 and the receptor-binding and translocation subunits of diphtheria toxin, was employed (Aullo *et al*., 1993). Another chimeric toxin was constructed of *C. botulinum* C2 toxin and C3 transferase. C3 was fused to the N-terminal part of the enzyme component of C2I, which is apparently involved in the interaction of C2I with C2II (Barth *et al*., 1998). This C2I-C3N fusion toxin could be introduced into cells via the binding and translocation component C2II (Figure 59.4). Similarly,

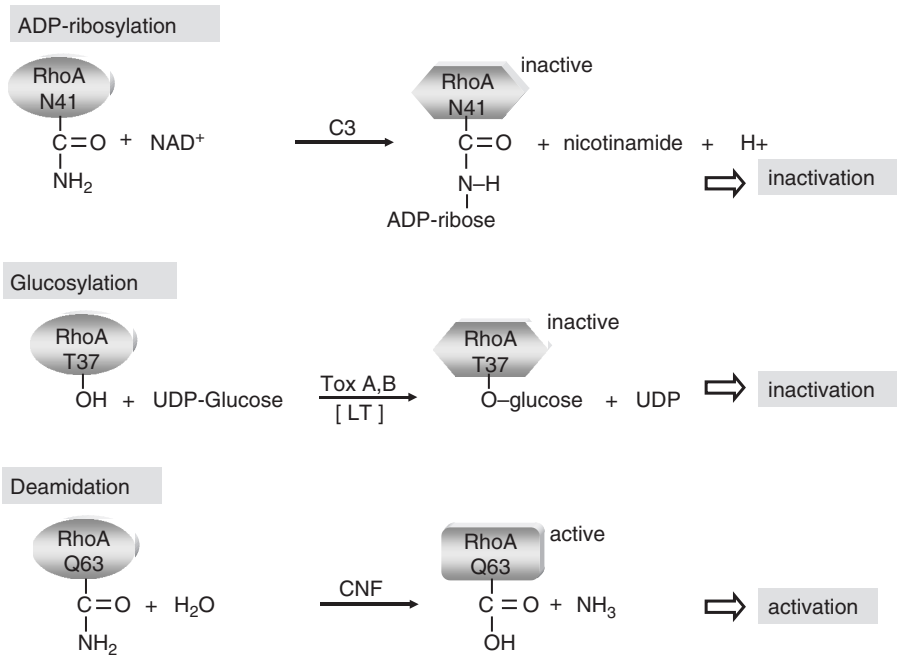



**FIGURE 59.2** Inactivation and activation of low molecular mass GTPases of the Rho family by various toxins often used as tools.

C3 exoenzymes specifically ADP-riboylate RhoA, B, and C. Thereby, Rho proteins (RhoA) are inactivated. Clostridial glucosylating toxins, e.g., *C. difficile* toxin A and B (ToxA, B) glucosylate many Rho proteins, including RhoA, Rac, and Cdc42. *C. sordellii* lethal toxin (LT) glucosylate Rac but not RhoA. In addition, Ras subfamily proteins are modified. Glucosylation blocks the biological activity of Rho GTPases.

*E. coli* cytotoxic necrotizing factor (CNF) deamidates Rho GTPases at glutamine63/61. This causes constitutive activation of the Rho proteins.

as observed with the diphtheria toxin-C3 fusion protein, in intact cells, the Rho-ADP-ribosylating activity of C2I-C3 was increased several hundredfold compared with that of the native C3 transferase. After removal of the chimeric toxin from the medium, cells recover rapidly after a few hours, most likely by neosynthesis of Rho, because the toxin is degraded rapidly (Barth *et al*., 1999). C3 from *C. botulinum* is translocated into cells by adding short peptides to the C-terminal end of the exoenzyme. For this purpose, short sequences of the human immunodeficiency virus transcription activator Tat are used (Sauzeau *et al*., 2001; Park *et al*., 2003). Moreover, the transport of C3 into cells can also be accomplished by fusing the third helix of the *Antennapedia* homoedomain protein from *Drosophila* to C3. In addition, short proline-rich peptides and highly basic arginine-rich peptides were C-terminally fused to C3 exoenzyme to facilitate the uptake of the transferase (Winton *et al*., 2002).

The intracellular expression of the C3 gene is another method to use C3-specific inhibition of Rho GTPases (Hilal-Dandan *et al*., 2004). Transgenes based on the thymocyte specific lck promoter have been used for expression of C3 in thymus (Henning *et al*., 1997). Recently, a transgenic mouse model expressing C3 exoenzyme in a lens-specific manner was utilized (Maddala *et al*., 2004). Under transcriptional control of the lens-specific alpha A-crystallin promoter, mice, expressing the C3 exoenzyme transgene, exhibited selective ocular defects (Rao *et al*., 2002).

As mentioned above, the use of C3 was most important in elucidating the functions of Rho proteins. Inhibition of a specified signaling pathway by using C3 was taken as an indication that Rho is involved in the signaling process. Thereby, pioneering work on the role of RhoA was performed showing that RhoA specifically controls stress fiber formation and induction of cell adhesions (Ridley and Hall, 1992). C3 was frequently used as a tool to study the role of Rho in regulation of the actin cytoskeleton, in cell motility, migration, and cell invasion. For example, C3 was used to study the role of Rho in translocation of aquaporin-2 into the apical membrane of renal cells induced by vasopressin (Klussmann *et al*., 2001). The toxin was successfully applied in studies on the regulatory function of Rho GTPases in neurite outgrowth, branching, and neuroregeneration (Lehmann *et al*., 1999; Wahl *et al*., 2000). Similarly, the role of Rho GTPases in the control of phospholipase D and in phospholipid metabolism was successfully studied with C3 (Meacci *et al*., 1999). Functions of Rho GTPases in transcriptional activation were another important topic, which was frequently addressed by applying C3 as a tool (Alberts and Treisman, 1998). Moreover, C3 was successfully employed in delineation of the role of Rho in the signaling of various heptahelical receptors to the actin cytoskeleton, phospholipases, and the nucleus via heterotrimeric G proteins. Especially important was C3 in studies on the functions of $G\alpha_{12/13}$ (Mao *et al*., 1998). Finally, C3 was studied in cell division and apoptosis (Kishi *et al*., 1993; Fiorentini *et al*., 1998).

## Clostridial glucosylating cytotoxins

Rho proteins are the targets of various clostridial cytotoxins, which modify the GTPases by glucosylation (Just *et al*., 1995; Just and Gerhard, 2004). Having molecular masses between 250 and 308 kDa, these toxins have been termed "large" clostridial cytotoxins. However, the name *clostridial glucosylating toxins* appears to be more informative and will be used here. *C. difficile* toxins A and B, which are the major virulence factors in antibiotic-associated diarrhea, are the most important members of this toxin family. Toxin B is termed cytotoxin, because it is several hundredfold more potent in inducing cytotoxic effects *in vitro* than toxin A. Whether the difference in potency is related to different cell membrane receptors is not clear. Both toxins have very similar lethal dose in mice (minimum lethal dose in mice about 50 ng i.p.).

*C. difficile* toxins A and B glucosylate Rho GTPases by using UDP-glucose as a second substrate (Figure 59.2). Not only RhoA, RhoB, and RhoC, but also other members of the Rho subfamily, like Cdc42 and Rac, are substrates. By contrast, small GTPases like Ras, Rab, Arf, or Ran are not substrates. The toxins glucosylate Thr37 of RhoA (Thr35 of Rac and Cdc42), which is involved in coordination of the magnesium ion and binding of nucleotides. Threonine 35/37 is located in the so-called switch-I region of the GTPases, which participates in effector coupling. This means that the region is responsible for the switch function of the GTPase in signaling. Toxin-catalyzed glucosylation inhibits the RhoA-effector interaction. This explains the functional inactivation of the GTPase after toxin treatment (Sehr *et al*., 1998). Notably, the acceptor amino acid residue for glucosylation is located very close to the site of ADP-ribosylation catalyzed by C3-like transferases (Asn41 in RhoA). This explains why glucosylated Rho is not modified by C3 and ADP-ribosylated Rho is not Substrate for clostridial glucosylating toxins.

*C. difficile* toxins A and B are often used as cell biological tools to screen the involvement of various Rho GTPases in a specific cellular process. For example, toxin B was used to study the involvement of Rho proteins in signal transduction of the FcεRI receptor and in histamine or serotonin secretion from mast cells or RBL cells (Prepens *et al*., 1996) (Figure 59.3). However, one

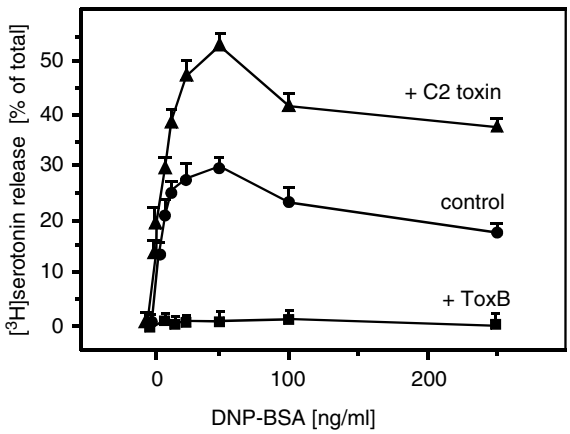



**FIGURE 59.3** Effects of *C. difficile* toxin B and *C. botulinum* C2 toxin on serotonin release from rat basophilic leucemia (RBL) cells. Serotonin release is induced by stimulation of the high-affinity antigen receptor (FcεRI) of RBL cells by increasing concentrations of the antigen DNP-BSA. Treatment with toxin B inhibits serotonin release, indicating the involvement of Rho GTPases in the degranulation response. The effect of toxin B is not caused merely by destruction of the actin cytoskeleton, because C2 toxin, which depolymerizes the actin cytoskeleton, does not block but increases serotonin release. (Data from Prepens *et al*., 1996)

has to consider that Rho proteins are involved in various signaling pathways, including regulation of the actin cytoskeleton. In the above-mentioned example, it is important to test whether the inhibitory effect of the toxin on secretion is a primary effect or secondary to depolymerization of the actin cytoskeleton. Another agent or toxin that selectively depolymerizes the actin cytoskeleton without having major effects on Rho proteins, such as cytochalasin or *C. botulinum* C2 toxin (see below), could be employed. In respect to histamine or serotonin secretion from RBL cells mentioned above, it was shown that C2 toxin increased receptor-induced secretion. This indicated that the inhibitory action of toxin B on secretion was not caused by disturbing the actin cytoskeleton.

The other members of the family of clostridial glucosylating toxins differ in cosubstrate or protein substrate specificity from toxins A and B; however, the conserved Thr35/37 residue of the GTPases is modified in each case. *C. novyi* alpha-toxin modifies Rho subfamily proteins like *C. difficile* toxin, but catalyzes N-acetylglucosaminylation by using UDP-GlcNAc as a co-substrate. *C. sordellii* lethal toxin, which is about 90% similar to *C. difficile* toxin B, also uses UDP-glucose as a co-substrate, but differs in its protein targets. Whereas Rac is a very good substrate for this toxin, Rho is poorly modified (most probably not at all in intact cells). In addition, lethal toxin glucosylates and inactivates Ras subfamily proteins like Ras, Rap, and Ral (Popoff *et al*., 1996). In recent years, several toxin B isoforms have been described that show substrate specificity similar to lethal toxin from *C. sordellii* (Mehlig *et al*., 2001). All these toxins are used as tools to get hints about the involvement of specific types of Rho proteins in signaling processes.

## RHO-ACTIVATING TOXINS

Hydrolysis of bound GTP is the switch-off mechanism of Rho GTPases. Mutation of amino acid residues involved in GTP hydrolysis is a well-established method to construct constitutively active (also called "dominant active") Rho GTPases, which possess no or marginal GTP hydrolysis activity. Transfection of mutated genes or microinjection of mutant proteins are frequently used in studies to elucidate the physiological role of Rho or related GTPases (Paterson *et al*., 1990). Interestingly, bacterial protein toxins exist that are capable of forming persistently active Rho mutants by posttranslational modification. These toxins are CNF1, 2 from *E. coli* and CNFy from *Yersinia*. CNFs catalyze the deamidation of glutamine 63 of RhoA to glutamic acid (Schmidt *et al*., 1997; Flatau *et al*., 1997) (Figure 59.2). Because glutamic acid is not able to fulfill the function of glutamine, the intrinsic and GAP-stimulated GTPase activity of Rho is blocked. CNF not only deamidates Rho, but also deamidates Cdc42 and Rac. Due to Rho GTPase activation, CNFs cause stress fiber, microspikes, and lamellipodia formation. Multinucleation is another typical feature of CNF-treated cells. Similarly to CNFs, dermonecrotic toxin (DNT; 154 kDa) causes activation of Rho proteins. However, DNT does not only deamidate the crucial glutamine (e.g., Gln63 of Rho) residue, but causes attachment of polyamines onto this residue, and therefore acts like a transglutaminase (Masuda *et al*., 2000). Because DNT is more unstable than CNFs, the latter is more often used to activate Rho proteins (Chaves-Olarte *et al*., 2002; Hopkins *et al*., 2003). Effective activation of Rho GTPases by the toxins can be monitored by different migration of deamidated RhoA in SDS-PAGE. By contrast, deamidated Rac and Cdc42 do not show altered migration behavior in SDS-PAGE. Activation of Rho GTPases is also tested in pull-down experiments performed with Rho GTPase effectors (or the GTPase-binding domains of the effectors), which only bind to the activated forms of the GTPases. Recently, it was observed that activation of Rac (less of RhoA and Cdc42) by CNF causes increased proteasomal degradation of the GTPase (Doye *et al*., 2002). Therefore, signal pathways, which depend on active Rac, may be stimulated by CNFs for a limited period of time only and not persistently. This has consequences for the monitoring of the toxin effects on cells. Whereas treatment of cells

with CNFs results in a Rac-like phenotypes after 2–4 h, which is characterized by membrane ruffling and lamellipodi formation, after 8–24 h toxin-treated cells exhibit morphological features characterized by RhoA activation (e.g., strong stress fiber formation). This change in morphology is accompanied by a decrease in the amount of Rac protein determined by Western-blotting.

## ADP-RIBOSYLATING TOXINS TO STUDY ACTIN

Actin is a major structural protein of the cytoskeleton. It is an essential part of the contractile apparatus of skeletal muscle and is vital for all kinds of motile processes, including migration, phagocytosis, endocytosis, secretion, and intracellular transport. Actin and the actin cytoskeleton are targeted by various bacterial protein toxins. The toxins directly modify actin by ADP-ribosylation or indirectly alter the regulation of the actin cytoskeleton by interfering with switch proteins of the Rho family, which control the cytoskeleton. In the latter case the above-mentioned Rho proteins, which are also preferred targets of bacterial protein toxins, play a crucial role.

### *Clostridium botulinum* C2 toxin

Actin is ADP-ribosylated by *Clostridium botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin, and the ADP-ribosylating toxin from *C. difficile* (Barth *et al*., 2004). All these toxins consist of a separate enzyme and cell-binding/translocation subunit. These components are not linked by either covalent or noncovalent bonds. The binding components are proteolytically activated and form heptamers.

Most often, C2 toxin from *C. botulinum* and iota toxin from *C. perfringens* were used as pharmacological or cell biological tools (Figure 59.4). Studies require activation of the binding component by trypsin (Ohishi *et al*., 1980). Thereby, the ~80-kDa binding component (C2II) of C2 toxin is cleaved into the ~65-kDa active fragment (C2IIa), which forms heptamers (Barth *et al*., 2000). Membrane receptors of the heptamers on eukaryotic target cells are complex and hybrid carbohydrate structures, which, so far studied, have been found on all types of mammalian cells (Eckhardt *et al*., 2000). Cellular uptake of the enzyme component (C2I, 50 kDa) occurs from acidified endosomal compartments via C2IIa that insert into the membrane and form pores. In the cytosol, C2I ADP-ribosylates actin at arginine-177. All other toxins, including SpvB, appear to modify the same residue of actin. C2I ADP-ribosylates nonmuscle β/γ–actin but not α-actin isoforms. In contrast, *C. perfringens* iota toxin catalyzes the ADP-ribosylation of all mammalian actin isoforms known (Mauss *et al*., 1990). The modification by the toxin is highly specific. Neither G proteins, which are substrates for the arginine-modifying CT, nor other cytoskeletal elements, such as tubulin, are ADP-ribosylated.

ADP-ribosylation of actin has major functional consequences. ADP-ribosylation blocks polymerization of G-actin. Arg177, which is modified by the toxins, is located at an actin-actin contact site, and the attachment of ADP-ribose appears to hinder actin interaction. This also explains why G-actin but not polymerized F-actin is substrate for ADP-ribosylation. Surprisingly, ADP-ribosylated actin acts as a capping protein, which binds to the plus ends of actin filaments (Wegner and Aktories, 1988). At this site of the polar actin filaments, usually filament growth occurs. Thus, by binding to the plus ends, ADP-ribosylated actin is able to inhibit the polymerization of unmodified G-actin onto preformed filaments. The minus ends of actin filaments are not affected by ADP-ribosylated actin. Therefore, dissociation of monomers, which preferentially occurs at these ends of filaments, is not altered. Monomeric actin released is subsequently ADP-ribosylated by the toxin and trapped in the non-polymerizable form. ADP-ribosylation also affects interaction of actin with actin-binding proteins (e.g., nucleation activity of the gelsolin actin complex), which are important for actin dynamics. Altogether, ADP-ribosylation causes destruction of the cytoskeleton and major morphological changes of targeted cells, which are reflected by rounding up of cells, which is accompanied by depolymerization of F-actin and an increase in the amount of G-actin. Thus, the actin ADP-ribosylating toxins are powerful tools to depolymerize actin filaments in intact cells. Accordingly, the toxins are used to study the functions of actin in intact cells. Alternatively, cytochalasins are often employed to study cellular consequences of actin depolymerization (Cooper, 1987). A tool to induce actin polymerization is phalloidin, which, however, is hampered by its poor cell accessibility. Recently, jasplakinolide (Holzinger, 2001) and latrunculin (Spector *et al*., 1999), both low-molecular-mass toxins from marine sponges, emerged as new tools to polymerize actin and depolymerize actin, respectively.

The actin-depolymerizing C2 toxin was employed in many studies to investigate the role of actin in exocytosis. In many types of culture cells, C2 toxin causes an increase in hormone or neurotransmitter-stimulated exocytosis. This was shown for the release of steroids in Y-1 adrenal cells (Considine *et al*., 1992), for serotonin release in RBL cells (Prepens *et al*., 1996), for

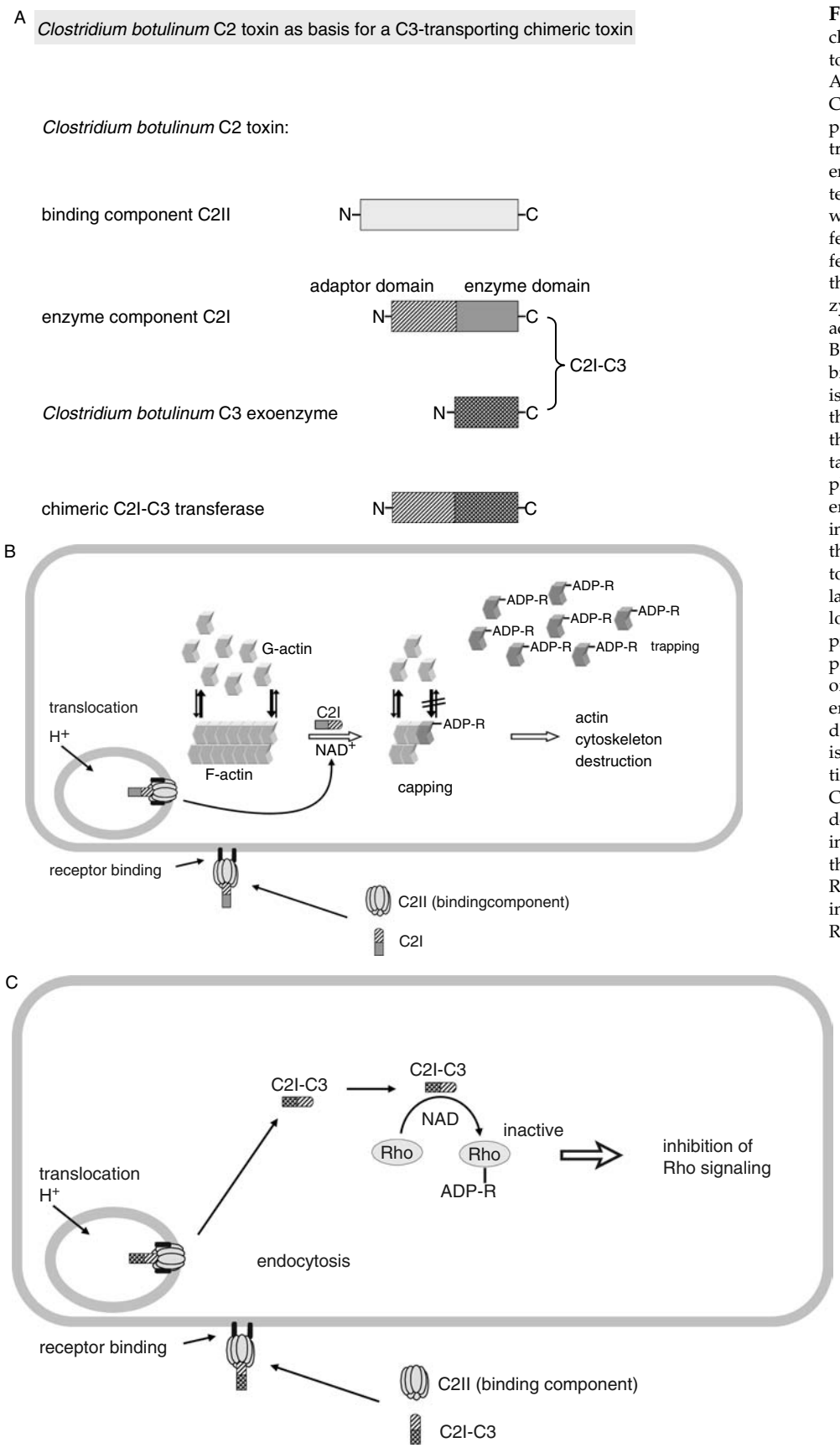



**FIGURE 59.4** Construction of a chimeric C3 toxin on the basis of C2 toxin.
A. The binary actin-ADP-ribosylating C2 toxin consists of the enzyme compoment (C2I) and the binding/translocation component C2II. The enzyme component of C2I has an N-terminal adaptor part for interaction with C2II and the C-terminal transferase part. The C-terminal transferase part of C2I is exchanged with the Rho-ADP-ribosylating exoenzyme C3, which possesses poor cell accessibility.
B. Action of C2 toxin in cells. The binding component C2II of C2 toxin is activated by trypsin treatment and then forms heptamers, which bind to the cell receptor. C2I binds to the heptamer and the receptor-toxin complex is endocytosed. In low pH endosomes, the binding component inserts into the membrane and allows the translocation of C2I into the cytotosol. In the cytosol C2I ADP-ribosylates actin. ADP-ribosylated actin has lost its ability to polymerize (trapping). Moreover, it acts like a capping protein to block the polymerization of G-actin at the fast growing "plus-ends" of actin filaments, while the depolymerization at the minus ends is not affected. This causes destruction of the actin cytoskeleton.
C. The chimeric C2I-C3 protein is delivered into the cytosol by the binding component C2II (see above). In the cytosol, C2I-C3 ADP-ribosylates Rho (e.g., RhoA). Thereby, Rho is inactivated, resulting in inhibition of Rho signaling pathways.

insulin release in isolated rat islets (Li *et al*., 1994), and for calcium-regulated exocytosis from adrenal chromaffin cells (Gasman *et al*., 1999). Usually, C2 toxin does not affect the basal release of mediators. Biphasic effects of the toxin were reported for the release of noradrenaline from PC12 cells (Matter *et al*., 1989). Short-term treatment (1 h) of the cells with C2-toxin increased noradrenaline release, whereas longer incubation decreased mediator release induced by depolarization or by carbachol. C2 toxin was used to study the role of actin in recycling of synaptic vesicles in the lamprey giant reticulospinal synapse (Shupliakov *et al*., 2002). It was found that C2 toxin inhibited endocytosis and changed the feature of clathrin-coated pits engaged in endocytosis. The data suggested that actin plays a role not only in vesicle transport at the synapse but also in the fission process.

In hamster insulinoma HIT-T15 cells, C2 toxin treatment inhibited insulin release by about 50%. Inhibition of insulin release was more pronounced in the second phase of the biphasic insulin release. These findings can be interpreted to indicate that actin filaments are involved in the recruitment of vesicles to the releasable pool and that C2 toxin blocks this action (Li *et al*., 1994). The toxin was employed to show the involvement of actin microfilaments in the retrograde transport from the Golgi to the endoplasmic reticulum (Valderrama *et al*., 2001). Also, in suspended mast cells (Prepens *et al*., 1998), C2 toxin inhibited regulated mediator release. In these cells, however, the inhibitory effect of C2 toxin turned into a stimulatory effect after adhesion of the mast cells, suggesting a complex role of actin, which cannot be explained simply by a barrier function or vesicle recruitment.

The actin-ADP-ribosylating C2 toxin was used to investigate the activation of neutrophils by chemotactic agents. In neutrophils, cell shape change, adhesion, migration, degranulation, and phagocytosis are induced by complement C5a, *N*-formyl peptides, or leukotriene $B_4$. It was shown by using the toxin that all these events depend on the redistribution of the cytoskeleton and on changes in actin polymerization (Norgauer *et al*., 1988). By using C2 toxin, it was shown that redistribution of the actin cytoskeleton largely affects the activity of superoxide anion-producing NADPH oxidase of neutrophils.

C2 toxin was used to investigate the role of actin in regulation of the serum responsive factor (Sotiropoulos *et al*., 1999). Quite often, C2 toxin was employed to study Rho signaling. In these cases, C2 toxin was used to distinguish effects merely caused by depolymerization of the actin cytoskeleton (e.g., caused by glucosylation of RhoA) from effects induced by Rho independently of the cytoskeleton. Appropriate controls of the specific action of C2 toxin are necessary in all these studies. Usually, the single toxin components (C2I or C2II) can be applied, which should not induce any effects in the system studied. It is also important that the toxin effects should occur with some delay of at least 15 to 30 min. This time is required for uptake of the toxin. It is recommended to test whether actin is actually ADP-ribosylated by the toxin. Moreover, the actions of C2 toxin should be compared with the effects of other actin-targeting agents, such as cytochalasins and latrunculin.

## CLOSTRIDIAL NEUROTOXINS AS TOOLS TO STUDY EXOCYTOSIS

Botulinum neurotoxins and tetanus toxin consist of a light chain (about 50 kDa), which is biologically active and harbors the enzyme activity, and a heavy chain (about 100 kDa), which is responsible for binding and membrane translocation. The chains are linked by a disulfide bond. The clostridial neurotoxins function as metalloendoproteases. The toxins selectively cleave synaptic proteins (synaptobrevin/VAMP, syntaxin, SNAP-25, and their respective isoforms), which are involved in the late steps of exocytosis and block neurotransmitter release from presynaptic nerve endings. One isoform of tetanus toxin (TeNT) and at least seven different serotypes of botulinum neurotoxins (BoNT/A, B, C1, D, E, F, and G) are known. Most importantly for the application as tools, the toxins differ in their protein substrate specificity, e.g., synaptobrevin is cleaved by tetanus toxin and BoNT/B, D, F, and G; SNAP25 is cleaved by BoNT/A, C, and E; and syntaxin is cleaved by BoNT/C. Moreover, most toxins cleave the synaptic proteins at different sites. Although TeNT and botulinum neurotoxins act on the molecular level in a very similar manner, their actions differ on the anatomic level. This is the reason why they cause completely different symptoms and diseases. Whereas botulinum neurotoxins act on peripheral nerves and cause flaccid paralysis, tetanus toxin acts on the central nervous system and induces spastic paralysis (tetanus) (Schiavo *et al*., 2000a; Rossetto *et al*., 2001).

Notably, the clostridial neurotoxins were of extreme importance for the elucidation of processes involved in neuronal exocytosis and vesicle-cell membrane fusion processes (Schiavo *et al*., 2000b). Moreover, in combination with permeabilization methods (e.g., usage of pore-forming toxins), clostridial neurotoxins are also used in studies on signal secretion coupling of non-neuronal cells (e.g., insulin-secreting cells) (Sadoul *et al*., 1995). These cells are otherwise insensitive toward neurotoxins because they lack the specific membrane

receptor. TeNT is also applied to induce experimental epilepsis (Bagetta and Nistico, 1994) and neuronal degeneration (Bagetta *et al*., 1991). Botulinum neurotoxins, which are the most potent toxic agents known, are now used as therapeutics. They are used, for example, to treat blepharospasm, strabism, and many different types of dystonias (Munchau and Bhatia, 2000), but also hyperhidrosis (Heckmann *et al*., 2001), pain from muscle spasm (Porta, 2000), and migraine headaches (Silberstein *et al*., 2000). Recently, the toxin is even used as a cosmetic (Blitzer and Binder, 2002).

## TOXINS FOR INTRACELLULAR PROTEIN DELIVERY

Intracellularly acting toxins possess the ability to translocate into cells. Although we have exciting new information about toxin translocation, this property is still largely enigmatic for many toxins. This is especially true for agents belonging to the biggest toxins known (e.g., large clostridial glucosylating cytotoxins with molecular masses of 250 to 308 kDa). The capability of protein toxins to cross the lipid cell membrane without major damage of the cell integrity has been exploited for use as a carrier system for proteins. Usually, these AB-toxins consist of the biologically active component (A, enzyme component) and the binding/translocation component (B). Considering that the binding and translocation domains are functionally and structurally separate entities, the toxins are better described as three-domain proteins. These functional domains are on a single toxin chain (e.g., *Pseudomonas* exotoxin A), on different chains linked by disulfide bonds (e.g., diphtheria toxin, botulinum neurotoxins), or they are on specific components, which are not covalently associated (e.g., CT and PT). Another type is the group of binary toxins, which have no linked enzyme and binding/translocation components. To construct a protein delivery system, all three toxin components may be used. To target a specific cell type, it might be necessary to change the receptor-binding part of the toxin. This is exemplified for diphtheria toxin and Pseudomonas exotoxin A immunotoxins, which were constructed to selectively target tumor cells. In the case of diphtheria toxin, the receptor-binding domain was exchanged with interleukine 2 to kill tumor cells (e.g., non-hodgkin cutaneous T cell lymphoma), which are rich in IL-2 receptors (Kreitman, 1999). Such toxins are already in clinical studies. Other toxin constructs are used to transfer an enzyme that otherwise is not able to cross the cell membrane into the cytosol. In this case, the receptor and translocation domains of the toxins are fused to the enzyme. The above-mentioned constructs of C3 with diphtheria toxin or with C2 toxin are examples (Figure 61.4). Also, the light chain of tetanus neurotoxin has been introduced into cells by means of an anthrax toxin fusion protein (Arora *et al*., 1994). Transport by anthrax toxin was also used to deliver cytoxic T cell epitopes into target cells to eventually stimulate specific cytotoxic T lymphocytes via MHC-1 complexes (Ballard *et al*., 1996).

## CONCLUSION

The previously mentioned examples show that various bacterial protein toxins are frequently and successfully employed as pharmacological and cell biological tools. In many studies, they were decisive instruments to elucidate the physiological functions of their target and turned out to be extremely helpful to clarify the cellular role of their mammalian substrates. These studies revealed that many bacterial protein toxins, and especially those that were earlier classified as "cytotoxins" because they appeared to alter the cell morphology in a uniform and "unspecific" manner, elicit highly specific effects. Knowledge of these subtle actions in target cells will certainly increase the usage of toxins as tools. Without question, the great potential of toxins as tools in cell biology and pharmacology is not yet fully exploited. An increase in our knowledge in their structure-function relationships will open new possibilities to use the bacterial protein toxins as pharmacological and cell biological tools, as well as therapeutic agents.

## REFERENCES

Ahnert-Hilger, G., Pahner, I. and Höltje, M. (2000). Pore-forming toxins as cell-biological and pharmacological tools. In: *Handbook of Experimental Pharmacology* (ed. K.Aktories and I.Just) **145,** 557–575.

Alberts, A.S. and Treisman, R. (1998). Activation of RhoA and SAPK/JNK signaling pathways by the RhoA-specific exchange factor mNET1. *EMBO J*. **17,** 4075–4085.

Aronson, A.I. and Shai, Y. (2001). Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett*. **195,** 1–8.

Arora, N., Williamson, L.C., Leppla, S.H. and Halpern, J.L. (1994). Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. *J. Biol. Chem*. **269,** 26165–26171.

Aullo, P., Giry, M., Olsnes, S., Popoff, M.R., Kocks, C. and Boquet, P. (1993). A chimeric toxin to study the role of the 21 kDa GTP binding protein Rho in the control of actin microfilament assembly. *EMBO J*. **12,** 921–931.

Bagetta, G. and Nistico, G. (1994). Tetanus toxin as a neurobiological tool to study mechanisms of neuronal cell death in the mammalian brain. *Pharmacol. Ther*. **62,** 29–39.

Bagetta, G., Nistico, G. and Bowery, N.G. (1991). Characteristics of tetanus toxin and its exploitation in neurodegenerative studies. *Trends Pharmacol. Sci.* **12,** 285–289.

Ballard, J.D., Collier, R.J. and Starnbach, M.N. (1996). Anthrax toxin-mediated delivery of a cytotoxic T cell epitope *in vivo. Proc. Natl. Acad. Sci. USA* **93,** 12531–12534.

Barth, H., Aktories, K., Popoff, M.R. and Stiles, B.G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. *Microbiol. Mol. Biol. Rev.* **68,** 373–402.

Barth, H., Blöcker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R. and Aktories, K. (2000). Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification. *J. Biol. Chem.* **275,** 18704–18711.

Barth, H., Hofmann, F., Olenik, C., Just, I. and Aktories, K. (1998). The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect. Immun.* **66,** 1364–1369.

Barth, H., Olenik, C., Sehr, P., Schmidt, G., Aktories, K. and Meyer, D.K. (1999). Neosynthesis and activation of Rho by *Escherichia coli* cytotoxic necrotizing factor (CNF1) reverse cytopathic effects of ADP-ribosylated Rho. *J. Biol. Chem.* **274,** 27407–27414.

Bhakdi, S., Valeva, A., Walev, I., Weller, U. and Palmer, M. (1997). Pore-forming toxins. In: *Bacterial Toxins—Tools in Cell Biology and Pharmacology.* ed. K.AktoriesWeinheim: Chapman & Hall, 241–257.

Bishop, A.L. and Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem. J.* **348,** 241–255.

Blitzer, A. and Binder, W.J. (2002). Cosmetic uses of botulinum neurotoxin type A: an overview. *Arch. Facial. Plast. Surg.* **4,** 214–220.

Cassel, D. and Pfeuffer, T. (1978). Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75,** 2669–2673.

Chaves-Olarte, E., Guzman-Verri, C., Meresse, S., Desjardins, M., Pizarro-Cerda, J., Badilla, J., Gorvel, J.P. and Moreno, E. (2002). Activation of Rho and Rab GTPases dissociates Brucella abortus internalization from intracellular trafficking. *Cell Microbiol.* **4,** 663–676.

Considine, R.V., Simpson, L.L. and Sherwin, J.R. (1992). Botulinum $C_2$ toxin and steroid production in adrenal Y-1 cells: The role of microfilaments in the toxin-induced increase in steroid release. *J. Pharmacol. Exp. Ther.* **260,** 859–864.

Cooper, J.A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105,** 1473–1478.

Doye, A., Mettouchi, A., Bossis, G., Clément, R., Buisson-Touati, C., Flatau, G., Gagnoux, L., Piechaczyk, M., Boquet, P. and Lemichez, E. (2002). CNF1 exploits the ubiquitin-proteasome machinery to restrict Rho GTPase activation for bacterial host cell invasion. *Cell* **111,** 553–564.

Eckhardt, M., Barth, H., Blöcker, D. and Aktories, K. (2000). Binding of *Clostridium botulinum* C2 toxin to asparagine-linked complex and hybrid carbohydrates. *J. Biol. Chem.* **275,** 2328–2334.

Fiorentini, C., Gauthier, M., Donelli, G. and Boquet, P. (1998). Bacterial toxins and the Rho GTP-binding protein: what microbes teach us about cell regulation. *Cell Death & Differentiation* **5,** 720–728.

Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C. and Boquet, P. (1997). Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387,** 729–733.

Gasman, S., Chasserot-Golaz, S., Popoff, M., Aunis, D. and Bader, M.F. (1999). Involvement of Rho GTPases in calcium-regulated exocytosis from adrenal chromaffin cells. *J. Cell Sci.* **112,** 4763–4771.

Genth, H., Gerhard, R., Maeda, A., Amano, M., Kaibuchi, K., Aktories, K. and Just, I. (2003). Entrapment of Rho ADP-ribosylated by *Clostridium botulinum* C3 exoenzyme in the Rho-GDI-1 complex. *J. Biol. Chem.* in press.

Gierschik, P., Sidiropoulos, D. and Jakobs, K.H. (1989). Two distinct Gi-proteins mediate formyl peptide receptor signal transduction in human leukemia (HL-60) cells. *J. Biol. Chem.* **264,** 21470–21473.

Gill, D.M. (1977). Mechanism of action of cholera toxin. *Adv. Cyclic Nucleotide Res.* **8,** 85–118.

Hayakawa, T., Takanaga, A., Tanaka, K., Maeda, S. and Seki, M. (2004). Distribution and ultrastructure of dopaminergic neurons in the dorsal motor nucleus of the vagus projecting to the stomach of the rat. *Brain Res.* **1006,** 66–73.

Heckmann, M., Ceballos-Baumann, A.O. and Plewig, G. (2001). Botulinum toxin A for axillary hyperhidrosis (excessive sweating). *N. Engl. J. Med.* **344,** 488–493.

Henning, S.W., Galandrini, R., Hall, A. and Cantrell, D.A. (1997). The GTPase Rho has a critical regulatory role in thymus development. *EMBO J.* **16,** 2397–2407.

Hilal-Dandan, R., Means, C.K., Gustafsson, A.B., Morissette, M.R., Adams, J.W., Brunton, L.L. and Heller, B.J. (2004). Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. *J. Mol. Cell Cardiol.* **36,** 481–493.

Holzinger, A. (2001). Jasplakinolide. An actin-specific reagent that promotes actin polymerization. *Methods Mol. Biol.* **161,** 109–120.

Hopkins, A.M., Walsh, S.V., Verkade, P., Boquet, P. and Nusrat, A. (2003). Constitutive activation of Rho proteins by CNF-1 influences tight junction structure and epithelial barrier function. *J. Cell Sci.* **116,** 725–742.

Just, I. and Gerhard, R. (2004). Large clostridial cytotoxins. *Rev. Physiol Biochem. Pharmacol.* **152,** 23–47.

Just, I., Selzer, J., Wilm, M., Von Eichel-Streiber, C., Mann, M. and Aktories, K. (1995). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* **375,** 500–503.

Kaper, J.B., Morris, J.G., Jr. and Levine, M.M. (1995). Cholera. *Clin. Microbiol. Rev.* **8,** 48–86.

Kishi, K., Sasaki, T., Kuroda, S., Itoh, T. and Takai, Y. (1993). Regulation of cytoplasmic division of *Xenopus* embryo by *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI). *J. Cell Biol.* **120,** 1187–1195.

Klussmann, E., Tamma, G., Lorenz, D., Wiesner, B., Maric, K., Hofmann, F., Aktories, K., Valenti, G. and Rosenthal, W. (2001). An inhibitory role of Rho in the vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. *J. Biol. Chem.* **276,** 20451–20457.

Kreitman, R.J. (1999). Immunotoxins in cancer therapy. *Curr. Opin. Immunol.* **11,** 570–578.

Lax, A.J., Pullinger, G.D., Baldwin, M.R., Harmey, D., Grigoriadis, A.E. and Lakey, J.H. (2004). The pasteurella multocida toxin interacts with signaling pathways to perturb cell growth and differentiation. *Int. J. Med. Microbiol.* **293,** 505–512.

Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebock, A., Leclerc, N., Tigyi, G. and McKerracher, L. (1999). Inactivation of Rho signaling pathway promotes CNS axon regeneration. *Journal of Neuroscience* **19,** 7537–7547.

Li, G., Rungger-Brändle, E., Just, I., Jonas, J.C., Aktories, K. and Wollheim, C.B. (1994). Effect of disruption of actin filaments by *Clostridium botulinum* C2 toxin on insulin secretion in HIT-T15 cells and pancreatic islets. *Mol. Biol. Cell* **5,** 1199–1213.

Maddala, R., Deng, P.F., Costello, J.M., Wawrousek, E.F., Zigler, J.S. and Rao, V.P. (2004). Impaired cytoskeletal organization and

membrane integrity in lens fibers of a Rho GTPase functional knockout transgenic mouse. *Lab Invest* **84,** 679–692.

Madden, J.C., Ruiz, N. and Caparon, M. (2001). Cytolysin-mediated translocation (CMT): a functional equivalent type III secretion in Gram-positive bacteria. *Cell* **104,** 143–152.

Mao, J., Xie, W., Yuan, H., Simon, M.I., Mano, H. and Wu, D. (1998). Tec/Bmx non-receptor tyrosine kinases are involved in regulation of Rho and serum response factor by Ga12/13. *EMBO J.* **17,** 5638–5646.

Masuda, M., Betancourt, L., Matsuzawa, T., Kashimoto, T., Takao, T., Shimonishi, Y. and Horiguchi, Y. (2000). Activation of Rho through a cross-link with polyamines catalyzed by *Bordetella* dermonecrotizing toxin. *EMBO J.* **19,** 521–530.

Matter, K., Dreyer, F. and Aktories, K. (1989). Actin involvement in exocytosis from PC12 cells: studies on the influence of botulinum C2 toxin on stimulated noradrenaline release. *J. Neurochem.* **52,** 370–376.

Mauss, S., Chaponnier, C., Just, I., Aktories, K. and Gabbiani, G. (1990). ADP-ribosylation of actin isoforms by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin. *Eur. J. Biochem.* **194,** 237–241.

Meacci, E., Vasta, V., Moorman, J.P., Bobak, D.A., Bruni, P., Moss, J. and Vaughan, M. (1999). Effect of Rho and ADP-ribosylation factor GTPases on phospholipase D activity in intact human adenocarcinoma A549 cells. *J. Biol. Chem.* **274,** 18605–18612.

Mehlig, M., Moos, M., Braun, V., Kalt, B., Mahony, D.E. and Von Eichel-Streiber, C. (2001). Variant toxin B and a functional toxin A produced by *Clostridium difficile* C34. *FEMS Microbiol. Lett.* **198,** 171–176.

Munchau, A. and Bhatia,K.P. (2000). Uses of botulinum toxin injection in medicine today. *BMJ* **320,** 161–165.

Murphy, A.C. and Rozengurt, E. (1992). *Pasteurella multocida* toxin selectively facilitates phosphatidylinositol 4,5-bisphosphate hydrolysis by bombesin, vasopressin, and endothelin. Requirement for a functional G protein. *J. Biol. Chem.* **267,** 25296–25303.

Nobes, C.D. and Hall, A. (1995). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81,** 53–62.

Norgauer, J., Kownatzki, E., Seifert, R. and Aktories, K. (1988). Botulinum C2 toxin ADP-ribosylates actin and enhances $O_2^-$ production and secretion but inhibits migration of activated human neutrophils. *J. Clin. Invest.* **82,** 1376–1382.

Nürnberg, B. (1997). Pertussis toxin as a cell biological tool. In: *Bacterial Toxins—Tools in Cell Biology and Pharmacology* (ed. K. Aktories), pp. 47–60. Chapman & Hall, Weinheim.

Ohishi, I., Iwasaki, M. and Sakaguchi, G. (1980). Purification and characterization of two components of botulinum C2 toxin. *Infect. Immun.* **30,** 668–673.

Palmer, M. (2001). The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon* **39,** 1681–1689.

Park, J., Kim, J.S., Jung, K.C., Lee, H.J., Kim, J.I., Kim, J., Lee, J.Y., Park, J.B. and Choi, S.Y. (2003). Exoenzyme Tat-C3 inhibits association of zymosan particles, phagocytosis, adhesion, and complement binding in macrophage cells. *Mol. Cells* **16,** 216–223.

Paterson, H.F., Self, A.J., Garrett, M.D., Just, I., Aktories, K. and Hall, A. (1990). Microinjection of recombinant $p21^{rho}$ induces rapid changes in cell morphology. *J. Cell Biol.* **111,** 1001–1007.

Payling-Wright, G. (1955). The neurotoxins of *Clostridium botulinum* and *Clostridium tetani. Pharmacol.Rev.* **7,** 413–465.

Plant, A. and Williams,N.A. (2004). Modulation of the immune response by the cholera-like enterotoxins. *Curr. Top. Med. Chem.* **4,** 509–519.

Popoff, M.R. *et al.* (1996). Ras, Rap, and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. *J. Biol. Chem.* **271,** 10217–10224.

Porta, M. (2000). A comparative trial of botulinum toxin type A and methylprednisolone for the treatment of myofascial pain syndrome and pain from chronic muscle spasm. *Pain* **85,** 101–105.

Prepens, U., Barth, H., Wilting, J. and Aktories, K. (1998). Influence of *Clostridium botulinum* C2 toxin on FceRI-mediated secretion and tyrosine phosphorylation in RBL cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **357,** 323–330.

Prepens, U., Just, I., Von Eichel-Streiber, C. and Aktories, K. (1996). Inhibition of FceRI-mediated activation of rat basophilic leukemia cells by *Clostridium difficile* toxin B (monoglucosyltransferase). *J. Biol. Chem.* **271,** 7324–7329.

Rao, V., Wawrousek, E., Tamm, E.R. and Zigler S Jr (2002). Rho GTPase inactivation impairs lens growth and integrity. *Lab Invest.* **82,** 231–239.

Ridley, A.J. and Hall,A. (1992). The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70,** 389–399.

Rossetto, O., Seveso, M., Caccin, P., Schiavo, G. and Montecucco, C. (2001). Tetanus and botulinum neurotoxins: turning bad guys into good by research. *Toxicon* **39,** 27–41.

Sadoul, K., Lang, J., Montecucco, C., Weller, U., Regazzi, R., Catsicas, S., Wollheim, C.B. and Halban, P.A. (1995). SNAP-25 is expressed in islets of Langerhans and is involved in insulin release. *J. Cell Biol.* **128,** 1019–1028.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J., Scalbert, E., Pacaud, P. and Loirand, G. (2001). Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ. Res.* **88,** 1102–1104.

Schiavo, G., Matteoli, M. and Montecucco, C. (2000b). Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* **80,** 717–766.

Schiavo, G., Matteoli, M. and Montecucco, C. (2000a). Neurotoxins affecting neuroexocytosis. *Physiol Rev.* **80,** 717–766.

Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M. and Aktories, K. (1997). Gln63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor 1. *Nature* **387,** 725–729.

Schnepf, E., Crickmore, N., van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62,** 775–806.

Sehr, P., Joseph, G., Genth, H., Just, I., Pick, E. and Aktories, K. (1998). Glucosylation and ADP-ribosylation of Rho proteins—effects on nucleotide binding, GTPase activity, and effector-coupling. *Biochemistry* **37,** 5296–5304.

Shupliakov, O., Bloom, O., Gustafsson, J.S., Kjaerulff, O., Low, P., Tomilin, N., Pieribone, V.A., Greengard, P. and Brodin, L. (2002). Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. *Proc. Natl. Acad. Sci. USA* **99,** 14476–14481.

Silberstein, S., Mathew, N., Saper, J. and Jenkins, S. (2000). Botulinum toxin type A as a migraine preventive treatment. For the BOTOX Migraine Clinical Research Group. *Headache* **40,** 445–450.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal a-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Sotiropoulos, A., Gineitis, D., Copeland, J. and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. *Cell* **98,** 159–169.

Spector, I., Braet, F., Shochet, N.R. and Bubb, M.R. (1999). New anti-actin drugs in the study of the organization and function of the actin cytoskeleton. *Microscopy Research and Technique* **47,** 18–37.

Valderrama, F., Duran, J.M., Babia, T., Barth, H., Renau-Piqueras, J. and Egea, G. (2001). Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. *Traffic* **2,** 717–726.

Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes & Development* **11,** 2295–2322.

Van den Akker, F., Merritt, E.A. and Hol, W.G.J. (2000). Structure and function of cholera toxin and related enterotoxins. In: *Bacterial Protein Toxins* (ed. K. Aktories and I. Just) pp. 109–131. Springer, Berlin.

van der Goot, F.G. (2001). Pore-forming toxins. *Curr. Top. Microbiol. Immunol*. **257,** 1–166.

Wahl, S., Barth, H., Ciossek, T., Aktories, K. and Mueller, B.K. (2000). Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J. Cell Biol*. **149,** 263–270.

Walev, I., Bhakdi, S.C., Hofmann, F., Djouder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl. Acad. Sci. USA* **98,** 3185–3190.

Wegner, A. and Aktories,K. (1988). ADP-ribosylated actin caps the barbed ends of actin filaments. *J. Biol. Chem*. **263,** 13739–13742.

Wilde,C., Barth, H., Sehr, P., Han, L., Schmidt, M., Just, I. and Aktories, K. (2002). Interaction of the Rho-ADP-ribosylating C3 exoenzyme with RalA. *J. Biol. Chem*. **277,** 14771–14776.

Wilde, C., Chhatwal, G.S., Schmalzing, G., Aktories, K. and Just, I. (2001). A novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* modifying RhoE and Rnd3. *J. Biol. Chem*. **276,** 9537–9542.

Wilson, B.A. and Ho, M. (2004). Pasteurella multocida toxin as a tool for studying G(q) signal transduction. *Rev. Physiol Biochem. Pharmacol*. **152,** 93–109.

Wilson, B.A., Zhu, X., Ho, M. and Lu, L. (1997). *Pasteurella multocida* toxin activates the inositol triphosphate signaling pathway in Xenopus oocytes via $G_{qa}$-coupled phospholipase C-b1. *J. Biol. Chem*. **272,** 1268–1275.

Winton, M.J., Dubreuil, C.I., Lasko, D., Leclerc, N. and McKerracher, L. (2002). Charcterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. *J. Biol. Chem*. **277,** 32820–32829.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15,** 245–250.

Zywietz, A., Gohla, A., Schmelz, M., Schultz, G. and Offermanns, S. (2001). Pleiotropic effects of *Pasteurella multocida* toxin are mediated by Gq-dependent and -independent mechanisms. Involvement of Gq but not G11. *J. Biol. Chem*. **276,** 3840–3845.

CHAPTER

# 60

# Engineering of bacterial toxins for research and medicine

*Aurélie Perier, Alexandre Chenal, Aurélie Babon, André Ménez, and Daniel Gillet*

## INTRODUCTION

Bacterial toxins are proteins capable of achieving multiple remarkable tasks (Menetrey *et al*., 2005; Parker and Feil, 2005). They function as autonomous molecular devices, targeting specific cells in an organism, punching holes in their membranes, or modifying intracellular components. Intoxication processes involve highly specialized steps of great complexity. It is thus tempting for the biochemist, the protein engineer, the biotechnologist, or the medical scientist to exploit the sophisticated properties of bacterial toxins to design new toxin-derived molecules for research, biotechnology, or medical treatments. Some toxins or toxin subunits are used in their natural form as biochemical and cell biology tools or for the treatment of specific diseases. For instance, streptolysin O is used to punch transient holes into cells for the delivery of oligonucleotides (Broughton *et al*., 1997) or proteins (Fawcet *et al*., 1998; Walev *et al*., 2001) into their cytoplasm. Cholera toxin B pentamer is used as a marker of lipid rafts due to its binding specificity for gangliosides sequestered in these membrane microdomains (Harder *et al*., 1998). It is also used as an adjuvant for mucosal vaccines (Freytag and Clements, 2005). *Clostridium* toxins are used to study actin and G proteins (Richard *et al*., 1999); botulinum toxin is used in the treatment of dystonies (Jankovic, 2004). Also, natural bacterial toxins inactivated by chemical treatment are used as vaccines. These include diphtheria, tetanus, and pertussis toxins.

Besides the use of native toxins as tools, therapeutics, or vaccines, toxins or toxin fragments can be engineered or combined with other protein domains to build rationally new proteins with new defined activities. This review focuses on the vast possibilities offered by toxin engineering for the design of new tools and therapeutics. The applications of native toxins are described in other chapters of this book. Also, the mechanisms of action of the toxins used for engineering are not detailed here. The reader is invited to return to the corresponding chapters of this book to find all precisions on those mechanisms.

A brief overview of the properties of bacterial toxins helps to understand the fascinating possibilities they offer as building blocks to tailor new proteins with desired activities (Table 60.1). Toxins are usually secreted by bacteria in a soluble form. The toxins are then able to diffuse in the aqueous environment of body fluids such as digestive or respiratory secretions, interstitial fluid surrounding infected tissues, lymph, blood, etc. Some toxins are capable of crossing epithelial barriers or penetrating deeply inside organs. For instance, botulinum toxin crosses the digestive wall from the intestine lumen by transcytosis to the blood circulation, and tetanus toxin reaches inhibitory interneurons in the central nervous system by retrograde transport through peripheral neurons. Cell intoxication proceeds with a series of successive steps involving different domains or subunits of the toxin (Figure 60.1). Activation of the toxin by proteolytic cleavage of a terminal peptide, an interdomain loop, or even an intradomain site is necessary for many toxins. This process may involve bacterial or host proteases and may occur before or after binding of the toxin to the surface of target cells. It is likely that the need for an activation mechanism prevents the toxins from inter-

**TABLE 60.1** Properties of bacterial toxins that can be exploited for engineering

| Properties | Toxins concerned[a] |
|---|---|
| Diffusion in body fluids | Pore-forming[b] and intracellular[c] |
| Crossing of epithelial barriers | Botulinum toxins, some $AB_5$ toxins |
| Penetration into central nervous system | Tetanus |
| Proteolytic activation | Pore-forming[b] and intracellular[c] |
| Targeting of cell surface receptors | Pore-forming[b] and intracellular[c] |
| Action on the cell surface (permeabilization) | Pore-forming[b] |
| Translocation from the cell surface | *B. pertussis* adenylate cyclase |
| Internalization and intracellular trafficking | Intracellular[c] except *B. pertussis* adenylate cyclase |
| Conformational change upon oligomerization | Pore-forming[b], $A_nB_7$ toxins |
| Conformational change upon acidification | $AB_{RT}$ toxins, $A_nB_7$ toxins |
| Membrane penetration | Pore-forming[b], $AB_{RT}$, $A_nB_7$ toxins |
| Pore formation (large size, poor or no selectivity) | Pore-forming[b] (except RTX toxins) |
| Permeabilization | RTX toxins, some $AB_{RT}$ |
| Channel formation (small size, high selectivity, voltage gated) | $AB_{RT}$, $A_nB_7$ toxins and colicins |
| Translocation from cell compartments | Intracellular[c] except *B. pertussis* adenylate cyclase |
| Enzymatic activities (see Menetrey *et al*., 2005 for list) | Intracellular[c] |

[a]See Figure 60.1 and Menetrey *et al*., 2005 for nomenclature. [b]Pore-forming toxins. [c]Toxins with intracellular targets.

acting inappropriately with bacterial or host membranes. Bacterial toxins select their target cells by recognizing specific cell surface receptors. Most toxins recognize receptors found on any cell types, thus having a broad spectrum of targets, while others recognize receptors found on very specific cells, such as neurons (*Clostridium* neurotoxins) or dendritic cells of the immune system (*Bordetella pertussis* adenylate cyclase). Pore-forming toxins act directly at the cell surface, in most cases after oligomerization (Parker and Feil, 2005). Toxins with intracellular targets of the $AB_{RT}$ and $AB_5$ types act usually as individual molecules (monomers or preassembled oligomers), while toxins of the $A_nB_7$ type assemble as oligomers following binding to the cell surface (Menetrey *et al*., 2005). These toxins penetrate the cells by the internalization pathways

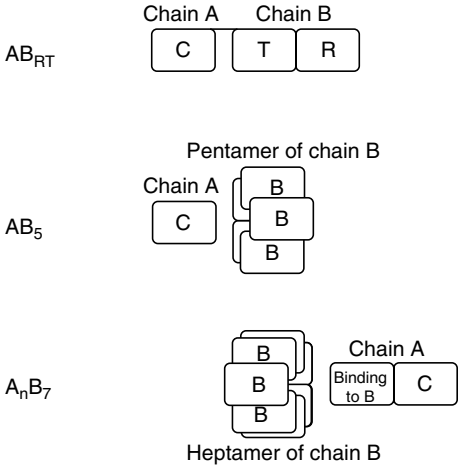



**FIGURE 60.1** Structure/function organization of toxins with intracellular targets (Ménétrey *et al*., 2005). In principle, each module (chain or domain) may be isolated and used for engineering in combination with other proteins. In all cases, chain A carries the catalytic domain (C) of the toxin. For $AB_{RT}$ toxins, chain B is formed of a receptor-binding domain (R) responsible for binding to the cell surface, receptor-mediated internalization, and intracellular trafficking and a translocation domain (T) responsible for passage of the C domain inside the cytoplasm. For $AB_5$ toxins, chain B is responsible for binding to the cell surface, receptor-mediated internalization, and intracellular trafficking. For $A_nB_7$ toxins, up to three A chains may bind to one B heptamer. Chain A contains a domain responsible for binding to the B heptamer and a C domain. Chain B is responsible for binding to the cell surface, receptor-mediated internalization, intracellular trafficking, and translocation of chain A into the cytoplasm. Among $AB_{RT}$ toxins are: diphtheria toxin *(C. diphtheria),* exotoxin A (*P. aeruginosa*), botulinum toxins (*C. botulinum*), tetanus toxin (*C. tetani*), large clostridial toxins (*C. difficile, C. sordellii, C. novyi*), and dermonecrotic toxins (*E. coli, B. pertussis*). The adenylate cyclase of *B. pertusis* resembles $AB_{RT}$ toxins, although its chain B is derived from an RTX toxin. Among $AB_5$ toxins are cholera toxin (*V. cholerae*), heat-labile toxins (*E. coli*), pertussis toxin (*B. pertussis*), Shiga toxin (*S. dysenteriae*), and Shiga-like toxins (*E. coli*). Among $A_nB_7$ toxins are anthrax toxin (*B. anthracis*), VIP toxin (*B. cereus*), C2 toxin (*C. botulinum*), iota toxin (*C. perfringens*), and actin-ADP-ribosylating toxins (*C. spiroforme* and *C. difficile*).

followed by their receptors. They are directed to given intracellular compartments depending on their receptors or on internal targeting sequences. Most toxins of both types (pore-forming or with intracellular targets) undergo major conformational changes involving one or several domains in order to interact with and penetrate into the membrane of the cell surface or of the intracellular compartments to which they are targeted. They create channels or pores, some with small sizes and narrow selectivity, some with very large sizes permeable to macromolecules. Toxins with intracellular targets translocate their catalytic domain (C) or subunit through the membrane of given cell compartments into the cell cytoplasm. This process involves major structural changes of the catalytic components (Falnes *et al*., 1994). Finally, these components refold in the cytoplasm and exert their enzymatic activities towards specific substrate molecules participating in key cellular processes (Menetrey *et al*., 2005).

All these steps, all these complex activities are frequently associated with individual domains, chains, or subunits of toxins (Figure 60.1). This is particularly true for toxins with intracellular activities. Many of these domains can be isolated from the rest of the toxin by recombinant DNA technology and used for their properties, isolated or combined with other proteins. Also, they can be modified by protein engineering. They may retain entirely or partially their original function, or even express new unexpected capabilities. Even genes encoding fragments of toxins or toxin receptors can be used to engineer cells or animals. Possibilities are infinite. This review intends to illustrate the concept of toxin engineering with a series of examples but it cannot be exhaustive. These examples are sorted according to the two principal types of bacterial toxins (toxins with intracellular activities and pore-forming toxins) and to the molecular functions or mechanisms that are exploited (Table 60.1). However, most examples of toxin engineering involve toxins with intracellular targets, especially diphtheria toxin, which is certainly one of the most extensively manipulated toxin (Chenal *et al*., 2002a) (Figure 60.2).

## ENGINEERING RECEPTOR-BINDING ACTIVITIES

### Tissue-specific cell ablation in mice by expression of a toxin receptor

Mice are not sensitive to diphtheria toxin, while humans are. This is due to differences in the sequence of the molecule acting as the receptor for the toxin in sensitive species (especially man and monkeys). The receptor for diphtheria toxin is a membrane precursor of an epidermal growth factor-like growth factor capable of binding heparin (proHB-EGF) (Naglich *et al*., 1992; Mitamura *et al*., 1995). Five residues of human proHB-EGF involved in toxin binding are mutated in the murine sequence, impairing recognition (Cha *et al*., 1998). Murine cells transfected with the simian gene for

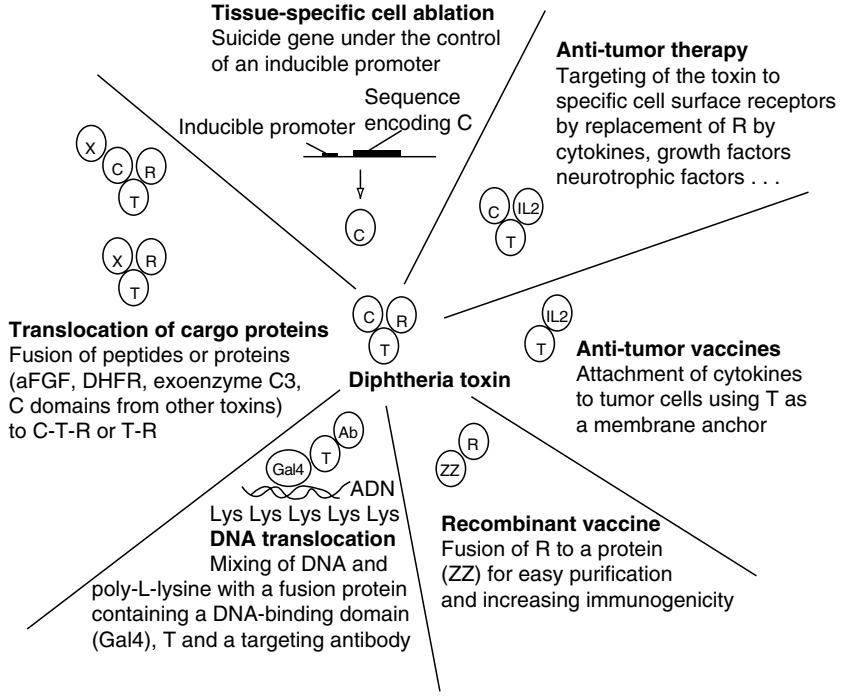



**FIGURE 60.2** Examples of diphtheria toxin engineering. See text for references.

proHB-EGF and expressing the receptor become sensitive to the toxin (Brown *et al*., 1993). On the basis of these data, Saito *et al*. (2001) demonstrated that human proHB-EGF can be used to sensitize specific cell types to diphtheria toxin in mice *in vivo*. This enables deletion of a given tissue or organ in order to study its function or treatments to supplement its loss. The gene for human proHB-EGF is placed under control of a promoter, whose expression is specific for the tissue to be deleted. The construct is introduced in mice embryos to produce transgenic mice. Only the tissue(s) corresponding to the tissue-specificity of the promoter expresses proHB-EGF. When needed, this tissue is destroyed by injection of diphtheria toxin to the mice. Using the liver cell–specific albumin promoter, mice developed fulminant hepatitis following administration of a small amount of diphtheria toxin (Saito *et al*., 2001).

## Toxoids based on receptor-binding domains of toxins

Vaccines against tetanus, diphtheria, and whooping cough are made of the corresponding toxins fixed with cross-linking agents such as formaldehyde. Anti-sera against tetanus or botulinum toxins are prepared by immunizing horses with such toxoids. Chemical cross-linking of residues separated in the primary sequence of the toxin but close in space due to the tertiary structure block the structural changes involved in the intoxication process (Paliwal and London, 1996). Large amounts of dangerous toxins must be produced to make toxoids. In addition, antibodies against toxoids could be of lesser quality than antibodies against the natural toxins. It has been proposed that protective antibodies are mostly directed against the receptor-binding domain of a toxin because they prevent the first step of intoxication, i.e., binding of the toxin to the target cells. As a consequence, several teams have used recombinant receptor-binding (R) domains of toxins as toxoids, in an isolated form, or fused to a protein increasing immunogenicity.

Recently, Tavallaie *et al*. (2004) defined the smallest part of botulinum toxin (type A) R domain (named fragment Hc) capable of generating a good protective immune response in mice. In fact, this part is the entire R domain itself. Each of the two subdomains defining the R domain is less efficient in generating a protective response, even when injected simultaneously. This indicates that the interface between both subdomains must carry important protective epitopes. Indeed, the structure of each isolated subdomain and of the entire R domain were studied by circular dichroism and fluorescence. The results show that the interface connecting the subdomains is highly structured, further suggesting that important epitopes defined by the tertiary structure of the interfacial region are necessary to elicit protective antibodies (Tavallaie *et al*., 2004). Overall, this work shows the potential of recombinant R domains as toxoids and the importance of conformational epitopes in generating a protective anti-toxin immune response.

In an attempt to make safe and easy-to-produce recombinant anti-diphtheria toxin vaccine, it has been proposed to link the R domain of diphtheria toxin to a protein that enables easy purification and that increases immunogenicity (Lobeck *et al*., 1998). The protein fused to R was ZZ, a derivative of the staphylococcal protein A made of two IgG-binding domains (Ljunberg *et al*., 1993; Jansson *et al*., 1998) (Figure 60.2). The presence of ZZ fused to R offers a simple and efficient means to purify the protein from bacterial extracts, using IgG affinity chromatography. Also, ZZ often increases immunogenicity of the fusion partner, presumably by enhancing its presentation to specific T cells. The fusion protein was able to generate antibodies inhibiting diphtheria toxin cytotoxicity. However, the antibody titers obtained with the recombinant protein were lower than with the formaldehyde-treated toxoid (Lobeck *et al*., 1998).

## Changing R domains of toxins to target toxicity to specific cell types

About a century ago Paul Erlich dreamed of ways to drive toxins to kill cancer cells while avoiding normal cells (Erlich, 1956). He called these targeted toxins "magic bullets." This idea started to concretize about 25 years ago when antibodies against cell surface markers were chemically conjugated to toxins (Trowbridge and Domingo, 1981). Exotoxin A from *Pseudomonas aeruginosa*, diphtheria toxin, and the plant toxin ricin were selected to build immunotoxins due to their high toxicity. For instance, one molecule of diphtheria toxin entering a cell is enough to kill it (Yamaizumi *et al*., 1978; Falnes *et al*., 2000). These immunotoxins were able to kill cells expressing the markers recognized by the antibodies. However, first-generation immunotoxins had many drawbacks. Full-size antibodies were used, leading to very large molecules diffusing poorly inside tissues. These antibodies from mice were highly immunogenic. The R domain of the toxin was not necessarily identified and removed, leading to unwanted killing of normal cells. The cloning of toxin genes and the increased knowledge of toxin structure progressively led to highly sophisticated engineered molecules (Kreitman and Pastan, 1995; Murphy and vanderSpek, 1995). Today, the same

three toxins are still used. In the case of diphtheria toxin (Figure 60.2) and *Pseudomonas* exotoxin A, the R domain is entirely eliminated. Only the C and T domains are kept, the later enabling the transfer of the former through the membrane of the compartment into which the toxin is internalized inside the cell cytoplasm. The C domains of both toxins ADP-ribosylate elongation factor 2, blocking protein synthesis of the cell. The R domain is replaced by a Fab, a single chain Fv fragment or a growth factor (Kreitman and Pastan, 1995; Murphy and vanderSpek, 1995). In most cases, whole immunotoxins are now prepared by gene fusion technology and recombinant protein expression. Chemical conjugation tends to be abandoned because recombinant proteins are more homogeneous, and orientation and accessibility of the binding site is easier to control. Growth factors or other receptor-binding ligands may be superior to antibody fragments when they have an affinity for their receptor higher than that of an antigen/antibody interaction. For instance, the dissociation constant of interleukine-2 for its high-affinity receptor is 10 picomolar, while that of an antibody for its antigen is rarely as good. In addition, recombinant single chain Fv fragments, which are fused to the toxin, are hard to produce and have frequently a reduced affinity for the antigen compared to the native antibody.

Since the publication in 1986 by John R. Murphy *et al*. of the first fusion protein connecting diphtheria toxin and a peptide hormone, the melanocyte-stimulating hormone (MSH), more than 20 diphtheria toxin-related fusion proteins have been described (Table 60.2). A whole set of cytokines and growth factors have been used that recognize a wide variety of cell types such as tumor cells, angiogenic cells, neurons, immune cells, etc. The derived immuno- or rather hormonotoxins are aimed at malignancies, autoimmune disease, pain, HIV infection, or graft-versus-host disease (Table 60.2). Two variants of a fusion toxin targeted to the receptor for interleukin-2 (IL-2) (Figure 60.2 and Table 60.2) have shown clinical activity in a variety of diseases, including cutaneous T cell lymphoma, B-cell non-Hodgkin's lymphoma, Hodgkin's disease, graft-versus-host disease, psoriasis, rheumatoid arthritis, and type I diabetes. The highest response rates were observed in cutaneous T cell lymphoma, leading to the approval of one of theses toxins by the United States Food and Drug Administration for the treatment of this disease as the drug denileukin diftitox (Ontak®) (Foss, 2000; Eklund and Kuzel, 2005). Clinical trials involving patients with acute myeloid leukemia have started to test a diphtheria toxin fusion protein targeted to the receptor for GM-CSF (Hall *et al*., 2001; Frankel *et al*., 2002). Other hormonotoxins derived from diphtheria toxin are under investigation for the treatment of various blood malignancies (Table 60.2). Although cutaneous T cell lymphoma, the target of Ontak, makes solid tumors, it is usually thought that circulating tumors should be more sensitive to immunotoxins because the toxins, which are administered intravenously, should reach the tumor cells more easily.

Unfortunately, the difficulties and costs linked to the development of such molecules and the clinical trials needed explain why only one fusion toxin already reached the pharmacy. Considering these difficulties, the experience gained during the development of Ontak helped to pinpoint critical points to address in order to make a drug from a targeted toxin. First, the fusion toxin must have a high affinity for its receptor, with $K_D$ close to picomolar values (Kiyokawa *et al*., 1989; Waters *et al*., 1990). This allows using low doses for the treatments, decreasing risks of side effects or non-specific toxicity. Second, the toxin must be devoid of residual binding to any normal tissue (Kuan *et al*., 1995; Siegall *et al*., 1997; Baluna *et al*., 1999). Third, the targeted cells must express high amounts of high-affinity receptors for the ligand linked to the toxin (Lakkis *et al*., 1991; vanderSpek *et al*., 1997), and these receptors must be efficiently internalized by the cells into the early endosomes, the compartment of translocation of diphtheria toxin (Lemichez *et al*., 1997). This may explain why Ontak is validated for cutaneous T cell lymphoma only and not for other lymphomas. Ideally, the efficacy of the treatment should be assayed *in vitro* on the tumor cells from each patient to assess good sensitivity of these cells.

In the late 1980s, a number of genetically engineered fusion toxins were described combining *Pseudomonas* exotoxin A and a series of targeting proteins such as IL-2, IL-4, IL-6, CD4, TGF alpha, and antibody Fv fragments (Chaudhary *et al*., 1988; Lorberboum-Galski *et al*., 1988; Siegall *et al*., 1988; Batra *et al*., 1989; Chaudhary *et al*., 1989; Ogata *et al*., 1989; Siegall *et al*., 1989). A toxin-Fv fragment fusion protein was targeted to CD25, one of the chains of the IL-2 receptor. Successive improvements were made to exotoxin A–derived immunotoxins (Brinkmann, 2000), and some encouraging responses were observed against lymphomas. Phase 2 trials are underway (Pastan, 2003). Many Fv fragments with other specificities targeting a variety of tumors were linked to exotoxin A. Among those is found an Fv fragment against CD22, a marker expressed on leukemia and lymphoma cells (Kreitman *et al*., 2001; FitzGerald *et al*., 2004). Unfortunately, immunotoxins derived from exotoxin A provoked vascular leak syndrome in patients, due to undesired toxicity toward endothelial cells (Kuan *et al*., 1995; Siegall *et al*., 1997; Baluna *et al*., 1999). In order to

**TABLE 60.2** Growth factors, peptide hormones, and protein domains fused to diphtheria toxin to target toxicity to specific tumors or tissues

| Targeting molecule | Targeted disease or tissue | Type of study | References |
|---|---|---|---|
| Human IL-2 | T lymphoma | *In vitro*, phase I and II, FDA-approved | Williams *et al*., 1987; Hesketh *et al*., 1993; LeMaistre *et al*., 1998, 2000; Foss *et al*., 2000; Eklund 2005 |
| | Graft versus host disease | Phase I | Ho *et al*., 2004 |
| | B-cell non-Hodgkin's lymphoma | Phase II | Dang *et al*., 2004 |
| | Rheumatoid arthritis | Rat, Phase II | Bacha *et al*., 1992; Moreland *et al*., 1995 |
| | Inflammatory bowel disease | *In vitro* | Bousvaros *et al*., 1997 |
| | Psoriasis | Phase I and II | Gottlieb *et al*., 1995; Bagel *et al*., 1998 |
| | Type 1 diabetes | Phase I/II | Woodworth and Nichols, 1993 |
| | HIV-infected cells | *In vitro* | Finberg *et al*., 1991 |
| Human IL-3 | Acute myeloid leukemia | *In vitro*, monkey | Frankel *et al*., 2000; Cohen *et al*., 2004 |
| Murine IL-3 | Myeloid leukemia | *In vitro*, mouse | Chan *et al*., 1996; Liger *et al*., 1997; Vallera *et al*., 1999; Black *et al*., 2003 |
| Murine IL-4 | Delayed-type hypersensitivity | *In vitro*, mouse | Lakkis *et al*., 1991 |
| Human IL-4 | Kaposi sarcoma | *In vitro* | Cai *et al*., 1997 |
| Human IL-6 | Myeloma, Kaposi sarcoma | *In vitro* | Chadwick *et al*., 1993a; Masood *et al*., 1994 |
| Human IL-7 | Leukemia | *In vitro* | Sweeney *et al*., 1998 |
| Human IL-13 | Gliobastoma, glioma | *In vitro*, mouse | Li *et al*., 2002; Tuo *et al*., 2004 |
| Human IL-13 + uPA | Glioblastoma | *In vitro* | Todhunter *et al*., 2004 |
| Simian IL-15 | T lymphoma | *In vitro* | vanderSpek *et al*., 1995 |
| scFv anti-CD3 | Graft versus host disease, T cell leukemia, activated T cells | *In vitro*, mouse, monkey | Ma *et al*., 1997; Vallera *et al*.,1997; Hubbard *et al*., 2001; Vallera *et al*., 2005 |
| CD4 | HIV-infected cells | *In vitro* | Aullo *et al*., 1992; Martin-Serrano *et al*., 1998 |
| EGF | Breast carcinoma, pancreatic carcinoma, glioblastoma | *In vitro*, mouse | LeMaistre *et al*., 1994; Mishra *et al*., 2003; Liu *et al*., 2005 |
| EGF-like domain of heregulin beta1 | Breast carcinoma | *In vitro* | Landgraf *et al*., 1998 |
| FGF6 | Kaposi, melanoma, breast, ovary, hepato-carcinoma | *In vitro* | Batoz *et al*., 1995 |
| Human G-CSF | Leukemia | *In vitro* | Chadwick *et al*., 1993b |
| Human GM-CSF | Acute myeloid leukemia | Monkey, phase I | Hotchkiss *et al*., 1999; Hall *et al*., 2001; Frankel *et al*., 2002; Abi-Habib *et al*., 2004; Cohen *et al*., 2004; Jedema *et al*., 2004 |
| GRP | Small cell pulmonary carcinoma | *In vitro* | vanderSpek *et al*., 1997 |
| MSH | Melanoma | *In vitro* | Murphy *et al*., 1986; Tatro *et al*., 1992 |
| Substance P | Pain | *In vitro*, rat | Fisher *et al*., 1996; Benoliel *et al*., 1999 |
| Tetanus toxin C fragment | Protein delivery to neurons | *In vitro* | Francis *et al*., 2000 |
| VEGF | Angiogenesis in solid tumors | Mouse | Olson *et al*., 1997; Arora *et al*., 1999; Hotz *et al*., 2002; Masood *et al*., 2003; Wild *et al*., 2004 |
| uPA | Acute myeloid leukemia | *In vitro* | Ramage *et al*., 2003 |
| Ovalbumin | Allergy to ovalbumin | Mouse | Lee *et al*., 2004 |

circumvent this serious side effect, the region of the toxin responsible for recognition of the endothelial cells was identified (Baluna *et al*., 1999). It seems to implicate an (x)D(y) tripeptide motif. This finding opens the way to additional engineering for safer immunotoxins.

### Neuron targeting and neuron-to-neuron delivery

The R domain of tetanus toxin, known as fragment C or TTC, corresponds to the C-terminal half of the heavy chain of the toxin. It has a molecular mass of 50 kDa. It binds specifically to neurons and may be used as a vector to target soluble proteins to the central nervous system. Like native tetanus toxin, fusion proteins or conjugates in which TTC is linked to a soluble protein show enhanced uptake and transport by motor neurons following intramuscular or systemic administration (Beaude *et al*., 1990; Fishman *et al*., 1990; Figueiredo *et al*., 1997). In addition, like the whole toxin, TTC enables transynaptic passage of passenger proteins from motor neurons to presynaptic processes and neurons (Fishman and Savitt, 1989; Beaude *et al*., 1990; Kissa *et al*., 2002). The aim of these studies is to deliver neurotrophic factors such as cardiotrophin-1 (Bordet *et al*., 2001) or lysosomal enzymes to the central nervous system for the treatment of neurodegenerative disorders. Also, a fusion protein in which green fluorescent protein is linked to TTC enables the visualization of synaptic transfer and neuronal networks *in vivo* (Kissa *et al*., 2002). Finally, a fusion protein containing the diphtheria toxin C and T domains linked to TTC has been constructed to combine the neuronal targeting property of TTC and the translocation property of diphtheria toxin (Francis *et al*., 2000). The aim is to deliver passenger proteins to the cytoplasm of neurons.

## ENGINEERING TOXIN ACTIVATION

During the intoxication of a cell, diphtheria toxin is activated by cleavage of a proteolytic site in a loop connecting the C and the T domain (Chenal *et al*., 2002a). This loop of 14 amino-acid residues is located between Cys 186 and Cys201, which form a disulfide bond linking the two domains after cleavage. The loop contains the sequence Arg190 Val191 Arg192 Arg193, which corresponds to a consensus motif recognized by the cellular protease furin and other proteases such as trypsin and PACE4. Cleavage takes place between Arg193 and Arg 194 (Tsuneoka *et al*., 1993). As furin is ubiquitously expressed, engineering of a tissue-specific protease cleavage site in place of the native activation site should enhance the selectivity of fusion toxins targeted to growth factor receptors. This principle has been tested on a diphtheria toxin GM-CSF fusion toxin targeted to acute myeloid leukemia cells (Abi-Habib *et al*., 2004). The sequence RVRRSV of the connecting loop was modified to a urokinase plasminogen activator (uPA) cleavage site GSGRSA. As a consequence, the toxin was highly toxic to leukemia cells expressing receptors for both GM-CSF and uPA. Toxicity correlated with expression levels of both receptors. Leukemia cells expressing GM-CSF receptors only were sensitized to the toxin by the addition of pro-uPA. Thus, normal myeloid stem cells expressing only the receptor for GM-CSF should not be sensitive to this toxin.

## EXPLOITING MEMBRANE BINDING AND TRANSLOCATION

Toxins with intracellular targets of the $AB_{RT}$ type (Menetrey *et al*., 2005) have a T domain, which assists the passage of the C domain through the cell membrane into the cytoplasm. Diphtheria, *Pseudomonas* exotoxin A, tetanus, and botulinum toxins are found among these toxins (Figure 60.1). T domains of known structure are made of alpha helices. They react at the acid pH of the endosome following internalization of the toxin. They adopt a molten globule conformation with loss of tertiary constraints, conservation of secondary structures, looser packing, and exposure of hydrophobic surfaces. This provokes the interaction of T with the membrane, its penetration into the bilayer, and a major reorganization of the helices (Gress *et al*., 1994; Chenal *et al*., 2002a, 2002b, 2003; Koriazova and Montal, 2003; Mere *et al*., 2005). The T domains of diphtheria and botulinum toxins form ion channels into membranes and at higher concentrations can destabilize and permeabilize lipid vesicles. Several applications have been found for the T domain of diphtheria toxin on the basis of its properties.

### Membrane anchors applied to cancer vaccination

Our group has shown that the T domain of diphtheria toxin can be used as a membrane anchor to attach soluble proteins to the surface of cells (Figure 60.2). Fusion proteins have been constructed in which the antibody-binding protein ZZ or cytokines [human IL-2, murine IL-3, and the Flt3 Ligand (FL)] have been fused to the N- or C-terminus of T (Liger *et al*., 1998; Nizard *et al*., 1998, 2001 and unpublished results). Incubation of cells with the

fusion proteins at acid pH (pH 4.8) drives the T domain to interact irreversibly with the membrane of the cells. In most cell types, the anchored proteins are not internalized. They remain displayed on the surface for several days. They can be recognized by specific antibodies and by cells carrying the corresponding receptor. These receptor-bearing cells respond to the signal found on the carrier cell. This stimulation is due to cell-cell contacts but also to a slow shedding of the anchored molecules in the culture medium (Nizard *et al*., 2003 and unpublished results). Such device allows the manipulation of the surface of given cells to modify their relations with other cells. It can be an advantageous alternative to gene transfection techniques. Indeed, many cell types are impossible to transfect efficiently, and it is hard to master the levels of expression of the transfected genes. In contrast, when using recombinant proteins connected to a membrane anchor and added externally to cells, it is possible to control the amounts attaching to the cell surface.

The membrane anchor principle has been used for the design of cancer vaccines assayed in mice. The studies were performed with two models of tumors, the RMA T cell lymphoma and the B16 melanoma. The vaccines were prepared by anchoring human IL-2 (Nizard *et al*., 2002; 2003) or human FL (unpublished results) to the surface of the cells using the T domain. The cells were previously treated with mitomycine to block their proliferation. Seventy percent of mice vaccinated with the RMA-IL-2 vaccine were fully protected against tumor challenge (Nizard *et al*., 2002, 2003). Protection was mediated by cytotoxic T lymphocytes specific for a tumor antigen. In mice vaccinated with the B16 vaccine, tumor progression was delayed. This result is significant since this tumor is poorly immunogenic. Vaccine efficacy depended on the precise dosage of cytokines anchored at the surface of tumor cells. Vaccination with a mixture of RMA cells carrying IL-2 and FL had a synergistic effect (unpublished observations). Overall, these vaccines were as efficient as similar vaccines prepared by transfer of cytokine genes inside tumors (Schmidt *et al*., 1995). However, preparation of the cells is easier using the membrane anchor technology. Also, this procedure presents several advantages as compared to gene transfection. It does not require the culture of tumor cells from the patients and eliminates the safety problems connected with viral vectors while allowing control of the amount of cytokine delivered with the vaccine (Nizard *et al*., 2002, 2003).

## Adjuvant for DNA transfection

The T domain from diphtheria toxin has been used as an adjuvant for DNA transfection. Passage of DNA particles from the compartments where they are internalized into the cell cytoplasm is rather inefficient. The T domain has been used in combination with DNA particles as a means to escape from these compartments because its interaction with membranes at acid pH can destabilize them. In a first study, T was chemically coupled to the high molecular mass poly-L-lysine used to complex the DNA. An asialo-oromucoid-polycation conjugate was incorporated to the complex to achieve targeting (Fisher *et al*., 1997). In two other studies, a fusion protein was constructed, which cumulated successively the DNA-binding domain of the yeast transcription factor Gal4, T, and either an antibody fragment specific for the tumor-associated ErbB2 antigen (Figure 60.2), or the tetanus toxin TTC fragment for cell targeting (Uherek *et al*., 1998; Barati *et al*., 2002). These proteins were associated within a complex of plasmid DNA and poly-L-lysine. In all cases, the presence of the T domain increased transfection efficacy.

## Translocation of cargo peptides and proteins

The capacity of translocation of bacterial toxins offers the fascinating perspective of transporting cargo proteins and peptides into targeted cells (Figure 60.2). Many studies have been done on the subject, but unfortunately, translocation is more complex than it seems and any protein cannot be translocated into a cell by a bacterial toxin. Several principles can be drawn from these studies. The translocation machinery of many toxins can translocate peptides up to 100 residues when fused to the C domain of that toxin (Stenmark *et al*., 1991; Madshus *et al*., 1992). The C domain can be mutated in its catalytic site to abolish its toxicity. The translocation machinery of many toxins can translocate the C domain of other toxins. Hence, it was shown that the B heptamer of anthrax toxin (the protective antigen heptamer) can translocate efficiently the C domain (or A chain) of diphtheria, *Pseudomonas*, tetanus, Shiga, or cholera toxins when fused to the B chain binding domain of lethal factor, one of the two genuine A components of anthrax toxin (see Figure 60.1) (Arora *et al*., 1992; Arora and Leppla, 1993, 1994; Milne *et al*., 1995; Sharma *et al*., 2000). Similarly, the C3 exoenzyme, an orphan enzymatic A chain possessing ADP-ribosyltransferase activity, can be translocated by the B chain of diphtheria toxin (Aullo *et al*., 1993) and the B heptamer of C2 and iota toxins (Barth *et al*., 1998; Marvaud *et al*., 2002). Also, the B chain of diphtheria toxin or *Pseudomonas* exotoxin A has been linked with the enzymatic component from ricin and the resulting chimeric toxins efficiently translocate the ricin enzymatic moiety (Sundan *et al*., 1982; Pitcher *et al*., 1995). Diphtheria toxin can translocate proteins if they are

highly unstable or unfolded. This was shown for dihydrofolate reductase (Klingenberg *et al*., 1996) and acidic fibroblast growth factor (Wiedlocha *et al*., 1992). Once stabilized by a ligand, these proteins were not translocated anymore. Thus, the data suggest that most proteins are incompetent or too stable to be translocated by toxins.

### Delivery of peptide or protein antigens to dendritic cells for vaccination

Toxins are now widely used for the preparation of experimental vaccines. The principle of these toxin-based vaccines is to favor the uptake of viral or tumor antigens or their fragments by the dendritic cells of the immune system. In fact, toxins are used as vectors to deliver these antigen fragments inside the dendritic cells. These cells are responsible for the onset of an immune response. Their role is to engulf necrotic or apoptotic cells in tissues, including virally infected cells or tumor cells. Then they traffic to lymph nodes and present the antigens contained in these dead cells to T lymphocytes. As a result, the lymphocytes are stimulated against the virally infected cells or the tumor cells. They leave the lymph node through the circulation to reach the periphery where they find the sick cells and destroy them. Manipulating the uptake of antigens by dendritic cells directly *in vivo*, or *ex vivo* before re-infusion to the patient, to produce strong anti-tumor immune responses has become a major area of research in immunotherapy. Among the toxins used for these studies are diphtheria toxin, *Pseudomonas* exotoxin A, the adenylate cyclase from *Bordetella pertussis*, Shiga toxin, pertussis toxin, anthrax toxin (Smith *et al*., 2002a) (and also the plant toxin ricin (Smith, 2002b), and the insect toxin phospholipase $A_2$ from bee venom (Babon *et al*., 2005)). In most studies, the antigenic peptides or protein fragments are fused to the C domain (or A chain) of the toxin. The C domain is mutated on its catalytic site to prevent toxicity. In the case of Shiga toxin, the A chain can be removed and antigenic sequences can be fused (Lee *et al*., 1998; Haicheur *et al*., 2000) or chemically coupled (Haicheur *et al*., 2003) directly to the B subunit. Some of these toxins have an enhanced specificity for dendritic cells, allowing immunization *in vivo* instead of *ex vivo*. The adenylate cyclase from *Bordetella pertussis* targets specifically the marker CD11d/CD18 expressed on dendritic cells, which is the alpha (M) beta (2) integrin (Guermonprez *et al*., 2001). Direct injection of the toxin-derived vaccine *in vivo* allows efficient stimulation of cytotoxic T lymphocytes (Saron *et al*., 1997; Fayolle *et al*., 2001). The receptor for Shiga toxin is the ganglioside Gb3, which is found on the surface of various cell types, but in high amounts on dendritic cells. The non-toxic B pentamer of this toxin has been used successfully as a vector for tumor antigen peptides fused to it (derived from MAGE-1 or the mouse mastocytoma P815) (Lee *et al*., 1998; Haicheur *et al*., 2000) or for a chemically coupled, full-size model antigen (ovalbumin) (Haicheur *et al*., 2003). Vaccination of mice with these constructions primed specific cytotoxic T lymphocyte responses without the use of adjuvant.

### Translocation of drugs to the nuclei of receptor-targeted cells

The development of some anti-tumor drugs is precluded by the difficulty of these compounds to penetrate inside tumor cells and reach their target. Fusion proteins were constructed with the aim to deliver photosensitizing drugs to the nucleus of melanoma cells (Rosenkranz *et al*., 2003). These proteins contained MSH as a melanoma cell targeting sequence, an optimized nuclear localization sequence of the SV40 large T-antigen, an *Escherichia coli* hemoglobin-like protein as a carrier for photosensitizing drugs, and the T domain of diphtheria toxin as a translocator. These modular transporters delivered the drugs into the nuclei of murine melanoma cells, but not into cells devoid of MSH receptors, thereby enhancing the potency of the drugs.

## ENGINEERING C DOMAINS

### Suicide gene encoding a C domain

Suicide genes can be made from the DNA sequence encoding the C domain (or A chain) of toxins with intracellular targets placed under the control of an inducible promoter. Such a gene was constructed with the sequence encoding the C domain of diphtheria toxin (Figure 60.2) under control of a promoter regulated by HIV. The gene transfected in human T and promonocytic cell lines stopped the spread of HIV infection on cell cultures *in vitro* (Dinges *et al*., 1995). However, protection depended on the stock of virus used. More interestingly, hematopoietic CD34+ stem cells from human cord were transduced with the HIV-regulated diphtheria toxin C domain gene and were used to reconstitute a human immune system in SCID mice (Banda *et al*., 1998). The CD4+ T cells were protected from challenge with HIV 44 days after engraftment in mice. This may be a promising approach for the treatment of AIDS by toxin gene therapy of bone marrow stem cells.

Suicide gene technology based on the C domain from diphtheria toxin can be applied to the ablation of

specific tissues in mice in order to provide models of degenerative diseases (Brockschnieder *et al*., 2004). A transgenic mouse strain was generated carrying a genetic construct for conditional expression of the C domain of diphtheria toxin. This construct is made by the introduction of the *lacZ* coding sequence flanked by *loxP* sites within the sequence of the C domain. The construct is introduced in a mouse locus ubiquitously expressed and as such expresses only beta-galactosidase as a control of expression. When this mouse strain is crossed with another transgenic mouse carrying a tissue-specific Cre recombinase gene, the recombinase excises the *lacZ* sequence from the C domain sequence through recognition of the *loxP* sites. This activation of the C domain sequence occurs only in the tissue expressing Cre. The C domain expression leads to destruction of the specific tissue. This system was applied to the specific ablation of hepatocytes, B cells, cortical neurons, and glial myelinating cells, respectively (Brockschnieder *et al*., 2004). Other studies using similar approaches exist. A model of basal ganglia disease has been described by expression of an attenuated form of the toxin gene in D1 dopamine receptor neurons (Padungchaichot *et al*., 2000; Wong *et al*., 2000). Transgenic mice expressing the C domain of diphtheria toxin under the control of the granzyme A promoter lead to specific ablation of CD8 T lymphocytes (Aguila *et al*., 1995).

### Double hybrid technology using the C domain of *B. pertussis* adenylate cyclase

*B. pertussis* adenylate cyclase may be considered as an $AB_{RT}$ toxin with a C domain located in the first 400 residues and a translocation and a receptor-binding activity carried by its 1,306 last residues. The region of the toxin carrying these two last functions is similar to RTX toxins. The C domain is activated by calmodulin, which binds with high affinity to the enzyme, but has a residual activity in the absence of calmodulin (Dautin *et al*., 2002). The C domain has a modular structure consisting of two complementary fragments: T25 (residues 1–224) and T18 (residues 225–399). Both fragments are necessary for enzymatic activity, but when co-expressed in *Escherichia coli*, they are unable to interact and have no activity. However, when T25 and T18 are fused to peptides or proteins able to interact, heterodimerization of the two hybrid polypeptides results in functional complementation of the two fragments of the C domain. The complex is able to synthesize cAMP. If expressed in a bacterial strain deficient for its endogenous adenylate cyclase, the enzymatic activity can be detected on an appropriate selective media. This bacterial double-hybrid screening system has been used successfully to detect a number of bacterial, yeast, viral, and human protein-protein interactions (see Dautin *et al*., 2002 for list). Similarly, this screening system can be used to study and select protease inhibitors in *Escherichia coli*. T25 and T18 can be connected together with a peptide linker carrying a proteolytic cleavage site, leading to a functional adenylate cyclase. In the presence of the protease, the enzymatic activity is lost. It is recovered in the presence of efficient protease inhibitors. This principle was tested with the human immunodeficiency virus protease and known inhibitors. It could distinguish wild-type protease from inhibitor-resistant proteases from infected patients resistant to antiretroviral therapy (Dautin *et al*., 2002).

## ENGINEERING OF ALPHA TOXIN FROM *STAPHYLOCOCCUS AUREUS*

Alpha-hemolysin from *Staphylococcus aureus* is a pore-forming toxin secreted as a 33-kDa monomer. After binding to the surface of cells, the monomers can self-assemble to form a heptamer. Then an important structural change occurs affecting a three-strand beta-sheet from each monomer, which reorganizes into a long two-strand beta-sheet plunging into the membrane (Song *et al*., 1996; Olson *et al*., 1999). Together, these beta-sheets from the seven subunits of the heptamer form a beta-barrel spanning the lipid bilayer, making a pore of about 2 nm in diameter and 10 nm long. The pore has a high conductance of about 700 pS and is permeable to molecules up to a molecular mass of 2,000. The group of Hagan Bayley at Texas A&M University has described ways to engineer this pore in order to use it as a device tunable by proteases or metal ions to control cell-membrane permeability, or as a sensor for organic molecules.

### Triggering of pore activity by a protease cleavage switch

Residue 131 of alpha-hemolysin is located at the tip of the long two-strand beta-sheet of each monomer forming the transmembrane stem of the pore. Fragments 1–131 and 132–293 of alpha-hemolysin can combine together and lead to a functional pore. If the C-terminus of fragment 1–131 or the N-terminus of fragment 132–293 is extended, the functional complementation is lost. A proteolytic site can be added between the peptide chain extension and the rest of the fragment to which it is connected. Cleavage by the corresponding protease restores complementation of both fragments, leading to a functional pore (Walker and Bayley, 1994). Thus, it is possible to generate pore-forming proteins

with a protease-activated trigger. Introduction of a combinatorial cassette allowed the selection of proteolytic cleavage sites that are highly susceptible to activation by cathepsin B, a protease that is secreted by certain metastatic tumor cells (Panchal *et al*., 1996). Toxins obtained by this procedure should be useful for the permeabilization of malignant cells, thereby leading directly to cell death or permitting destruction of the cells with drugs that are normally membrane impermeant.

### Control of pore activity by a metal ion switch

Residues 130–134 of alpha-hemolysin are located at the tip of the long two-strand beta-sheet of each monomer forming the transmembrane stem of the pore. When these residues are replaced by five histidines, the toxin retains its ability to form pores (Walker *et al*., 1994; Russo *et al*., 1997). However, this histidine stretch constitutes a divalent metal ion-binding site. Binding of $Zn^{2+}$ at a concentration of 10 μM completely blocks pore activity. Chelating agents such as EDTA restore pore permeability by removing free $Zn^{2+}$ in the solution. This permeabilizing system was applied to control the loading of cells by trehalose, a sugar used to improve the tolerance of mammalian cells to desiccation (Acker *et al*., 2003).

### Modulation of pore activity and sensing of organic compounds

The pore of staphylococcol alpha-hemolysin can accommodate deep inside its cavity a molecule of beta-cyclodextrin applied on its trans side (Gu *et al*., 1999). Beta-cyclodextrin is a ring-shaped cyclic molecule, comprising seven D-glucose units forming a hydrophobic cavity. The cavity can encapsulate organic molecules in aqueous solution. When beta-cyclodextrin (or other cyclodextrins) is lodged in the lumen of the alpha-hemolysin pore, the conductance of the pore is reduced from about 700 pS to 200 pS (depending on the membrane potential and ionic strength) (Gu *et al*., 1999), and the ionic charge and selectivity are modified (Gu *et al*., 2000a, 2000b). The cyclodextrin acts as a binding site for organic molecules, which further block the pore conductance to values and with residence times specific for each molecule, leading to specific signatures (Gu *et al*., 1999). The authors propose that the hemolysin-cyclodextrin complex can be used as a sensor for a variety of organic compounds. In addition, the selectivity of the pore formed by the hemolysin-cyclodextrin complex can be modulated by the use of different cyclodextrins of various chemical compositions (Gu *et al*. 2000a, 2000b).

The activity of the pore of alpha-hemolysin can also be controlled by introduction of mutations in the transmembrane beta-barrel. Depending on the mutations, the control of pore permeability may be specific for given molecules. Several mutants were constructed in which a ring of up to 14 arginines was introduced near the constriction in the transmembrane beta-barrel (Cheley *et al*., 2002). The permeability of one mutant was almost completely blocked by phosphate anions at pH 7.5, but not by other oxyanions. Another mutant had a high affinity for inositol 1,4,5-trisphosphate, an important cell-signaling molecule, second messenger for $Ca^{2+}$ mobilization, but not for other second messenger molecules. Such engineered pores can be useful components to build sensors of cell-signaling molecules.

## CONCLUSION

We have seen in this short overview the fascinating possibilities offered by bacterial toxins for the design of new proteins with new functions. We have seen also how animals expressing genes encoding a fragment of toxin or a toxin receptor can serve as models for degenerative diseases. The FDA has approved one engineered toxin targeted to an endogenous human receptor for the treatment of a lymphoma. New indications are under study and the number of patients who will benefit from this molecule should increase in the future. Many fusion toxins built on the same model and targeted to a variety of receptors are developed against a series of malignancies (Table 60.2). A few are now being evaluated in patients. There is little doubt that in the future, hospital pharmacies should possess complete sets of receptor-targeted toxins against a variety of leukemias, lymphomas, hopefully solid tumors, immunological disorders, etc. Toxins used as delivery vehicles for vaccines are starting to reach clinical trials. Many other engineered toxins are still in the test tubes. Some will remain as laboratory curiosities, while others may be future drugs. Some may become important biological tools for diagnostics, detectors, nano-technologies, or bioelectronics. A number of toxins are still undiscovered or poorly characterized and should offer new engineering possibilities. Important challenges are still in front of us: building of artificial channels and receptors; mastering injection of peptides, proteins, nucleic acids, or chemical compounds into cells; and targeting of substances to specific organs such as the central nervous system. Other possibilities are still "un-thought" of. However, the development of highly complex products from toxins for human applications is extremely long and costly. It took 15 to 20 years to

make a drug by engineering diphtheria toxin, about twice as long as for a conventional drug. It's just a beginning.

## REFERENCES


Abi-Habib, R.J., Liu, S., Bugge, T.H., Leppla, S.H. and Frankel, A.E. (2004). A urokinase-activated recombinant diphtheria toxin targeting the granulocyte-macrophage colony-stimulating factor receptor is selectively cytotoxic to human acute myeloid leukemia blasts. *Blood* **104,** 2143–2148.

Acker, J.P., Lu, X.M., Young, V., Cheley, S., Bayley, H., Fowler, A. and Toner, M. (2003). Measurement of trehalose loading of mammalian cells porated with a metal-actuated switchable pore. *Biotechnol. Bioeng*. **82,** 525–532.

Aguila, H.L., Hershberger, R.J. and Weissman, I.L. (1995). Transgenic mice carrying the diphtheria toxin A chain gene under the control of the granzyme A promoter: expected depletion of cytotoxic cells and unexpected depletion of CD8 T cells. *Proc. Natl Acad. Sci. USA* **92,** 10192–10196.

Arora, N., Klimpel, K.R., Singh, Y. and Leppla, S.H. (1992). Fusions of anthrax toxin lethal factor to the ADP-ribosylation domain of Pseudomonas exotoxin A are potent cytotoxins which are translocated to the cytosol of mammalian cells. *J. Biol. Chem*. **267,** 15542–15548.

Arora, N. and Leppla, S.H. (1993). Residues 1–254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides. *J. Biol. Chem*. **268,** 3334–3341.

Arora, N. and Leppla, S.H. (1994). Fusions of anthrax toxin lethal factor with Shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun*. **62,** 4955–4961.

Arora, N., Masood, R., Zheng, T., Cai, J., Smith, D.L. and Gill, P.S. (1999). Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. *Cancer Res*. **59,** 183–188.

Aullo, P., Alcami, J., Popoff, M.R., Klatzmann, D.R., Murphy, J.R. and Boquet, P. (1992). A recombinant diphtheria toxin-related human CD4 fusion protein specifically kills HIV infected cells, which express gp120, but selects fusion toxin-resistant cells, which carry HIV. *EMBO J*. **11,** 575–583.

Aullo, P., Giry, M., Olsnes, S., Popoff, M.R., Kocks, C. and Boquet, P. (1993). A chimeric toxin to study the role of the 21 kDa GTP-binding protein Rho in the control of actin microfilament assembly. *EMBO J*. **12,** 921–931.

Babon, A., Almunia, C., Boccaccio, C., Beaumelle, B., Gelb, M.H., Menez, A., Maillere, B., Abastado, J.P., Salcedo, M. and Gillet, D. (2005). Cross-presentation of a CMV pp65 epitope by human dendritic cells using bee venom PLA2 as a membrane-binding vector. *FEBS Lett*. **579,** 1658–1664.

Bacha, P., Forte, S.E., Perper, S.J., Trentham, D.E. and Nichols, J.C. (1992). Anti-arthritic effects demonstrated by an interleukin-2 receptor-targeted cytotoxin (DAB486IL-2) in rat adjuvant arthritis. *Eur. J. Immunol*. **22,** 1673–1679.

Bagel, J., Garland, W.T., Breneman, D., Holick, M., Littlejohn, T.W., Crosby, D., Faust, H., Fivenson, D. and Nichols, J. (1998). Administration of DAB389IL-2 to patients with recalcitrant psoriasis: a double-blind, phase II multicenter trial. *J. Am. Acad. Dermatol*. **38,** 938–944.

Baluna, R., Rizo, J., Gordon, B.E., Ghetie, V. and Vitetta, E.S. (1999). Evidence for a structural motif in toxins and interleukin-2 that may be responsible for binding to endothelial cells and initiating vascular leak syndrome *Proc. Natl Acad. Sci. USA* **96,** 3957–3962.

Banda, N.K., Akkina, R.K., Terrell, K., Shpall, E.J., Tomczak, J., Campain, J., Claman, H., Cagle, L. and Harrison, G.S. (1998). Diphtheria toxin A gene-mediated HIV-1 protection of cord blood-derived T cells in the SCID-hu mouse model. *J. Hematother*. **7,** 319–331.

Barati, S., Chegini, F., Hurtado, P. and Rush, R.A. (2002). Hybrid tetanus toxin C fragment-diphtheria toxin translocation domain allows specific gene transfer into PC12 cells. *Exp. Neurol*. **177,** 75–87.

Barth, H., Hofmann, F., Olenik, C., Just, I. and Aktories, K. (1998). The N-terminal part of the enzyme component (C2I) of the binary Clostridium botulinum C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect. Immun*. **66,** 1364–1369.

Batoz, M., Coll Fresno, P.M., Pizette, S., Raffioni, S., Birnbaum, D. and Coulier, F. (1995). A diphtheria toxin/fibroblast growth factor 6 mitotoxin selectively kills fibroblast growth factor receptor-expressing cell lines. *Cell Growth Differ*. **6,** 1143–1149.

Batra, J.K., Jinno, Y., Chaudhary, V.K., Kondo, T., Willingham, M.C., FitzGerald, D.J. and Pastan, I. (1989). Antitumor activity in mice of an immunotoxin made with anti-transferrin receptor and a recombinant form of Pseudomonas exotoxin. *Proc. Natl Acad. Sci. USA* **86,** 8545–8549.

Beaude, P., Delacour, A., Bizzini, B., Domuado, D. and Remy, M.H. (1990). Retrograde axonal transport of an exogenous enzyme covalently linked to B-IIb fragment of tetanus toxin. *Biochem. J*. **271,** 87–91.

Benoliel, R., Eliav, E., Mannes, A.J., Caudle, R.M., Leeman, S. and Iadarola, M.J. (1999). Actions of intrathecal diphtheria toxin-substance P fusion protein on models of persistent pain. *Pain* **79,** 243–253.

Black, J.H., McCubrey, J.A., Willingham, M.C., Ramage, J., Hogge, D.E. and Frankel, A.E. (2003). Diphtheria toxin-interleukin-3 fusion protein (DT(388)IL3) prolongs disease-free survival of leukemic immunocompromised mice. *Leukemia* **17,** 155–159.

Bordet, T., Castelnau-Ptakhine, L., Fauchereau, F., Friocourt, G., Kahn, A. and Haase, G. (2001). Neuronal targeting of cardiotrophin-1 by coupling with tetanus toxin C fragment. *Mol. Cell Neurosci*. **17,** 842–854.

Bousvaros, A., Stevens, A.C., Strom, T.B., Murphy, J. and Lamont, J.T. (1997). Interleukin-2 fusion protein (DAB389IL-2) selectively targets activated human peripheral blood and lamina propria lymphocytes. *Dig. Dis. Sci* **42,** 1542–1548.

Brinkmann, U. (2000). Recombinant antibody fragments and immunotoxin fusions for cancer therapy. *In Vivo* **14,** 21–27.

Brockschnieder, D., Lappe-Siefke, C., Goebbels, S., Boesl, M.R., Nave, K.A. and Riethmacher, D. (2004). Cell depletion due to diphtheria toxin fragment A after Cre-mediated recombination. *Mol. Cell. Biol*. **24,** 7636–7642.

Broughton, C.M., Spiller, D.G., Pender, N., Komorovskaya, M., Grzybowski, J., Giles, R.V., Tidd, D.M. and Clark, R.E. (1997). Preclinical studies of streptolysin-O in enhancing antisense oligonucleotide uptake in harvests from chronic myeloid leukemia patients. *Leukemia* **11,** 1435–1441.

Brown, J.G., Almond, B.D., Naglich, J.G. and Eidels, L. (1993). Hypersensitivity to diphtheria toxin by mouse cells expressing both diphtheria toxin receptor and CD9 antigen. *Proc. Natl Acad. Sci. USA* **90,** 8184–8188.

Cai, J., Zheng, T., Murphy, J., Waters, C.A., Lin, G.Y. and Gill, P.S. (1997). IL-4R expression in AIDS-KS cells and response to rhIL-4 and IL-4 toxin (DAB389-IL-4). *Invest. New Drugs* **15,** 279–287.

Cha, J.H., Brooke, J.S. and Eidels, L. (1998). Toxin-binding site of the diphtheria toxin receptor: loss and gain of diphtheria toxin binding of monkey and mouse heparin-binding, epidermal growth

factor-like growth factor precursors by reciprocal site-directed mutagenesis. *Mol. Microbiol*. **29,** 1275–1284.

Chadwick, D.E., Jean, L.F., Jamal, N., Messner, H.A., Murphy, J.R. and Minden, M.D. (1993a). Differential sensitivity of human myeloma cell lines and normal bone marrow colony-forming cells to a recombinant diphtheria toxin-interleukin 6 fusion protein. *Br. J. Haematol*. **85,** 25–36.

Chadwick, D.E., Williams, D.P., Niho, Y., Murphy, J.R. and Minden, M.D. (1993b). Cytotoxicity of a recombinant diphtheria toxin-granulocyte colony stimulating factor fusion protein on human leukemic blast cells. *Leuk. Lymphoma* **11,** 249–262.

Chan, C.H., Blazar, B.R., Greenfield, L., Kreitman, R.J. and Vallera, D.A. (1996). Reactivity of murine cytokine fusion toxin, diphtheria toxin390-murine interleukin-3 (DT390-mIL-3), with bone marrow progenitor cells. *Blood* **88,** 1445–1456.

Chaudhary, V.K., Mizukami, T., Fuerst, T.R., FitzGerald, D.J., Moss, B., Pastan, I. and Berger, E.A. (1988). Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hybrid protein. *Nature* **335,** 369–372.

Chaudhary, V.K., Queen, C., Junghans, R.P., Waldmann, T.A., FitzGerald, D.J. and Pastan, I. (1989). A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin. *Nature* **339,** 394–397.

Cheley, S., Gu, L.Q. and Bayley, H. (2002). Stochastic sensing of nanomolar inositol 1,4,5-trisphosphate with an engineered pore. *Chem. Biol*. **9,** 829–838.

Chenal, A., Nizard, P. and Gillet, D. (2002a). Structure and function of diphtheria toxin: from pathology to engineering. *J. Toxicol.-Tox. Rev*. **21,** 321–359.

Chenal, A., Savarin, P., Nizard, P., Guillain, F., Gillet, D. and Forge, V. (2002b). Membrane protein insertion regulated by bringing electrostatic and hydrophobic interactions into play. A case study with the translocation domain of the diphtheria toxin. *J. Biol. Chem*. **277,** 43425–43432.

Chenal, A., Babon, A., Perier, A., Nizard, P., Forge, V. and Gillet, D. (2003). The translocation domain from diphtheria toxin: interaction with membranes and application as a membrane anchor for cancer vaccines, In: *Recent Res. Devel. Biochem*. (ed. S.G. Pandalai), pp. 985–1000. Vol. 4. Research Signpost, Trivandrum, India

Cohen, K.A., Liu, T.F., Cline, J.M., Wagner, J.D., Hall, P.D. and Frankel, A.E. (2004). Safety evaluation of DT(388)IL3, a diphtheria toxin/interleukin 3 fusion protein, in the cynomolgus monkey. *Cancer Immunol. Immunother. Online*, Epub ahead of print.

Dang, N.H., Hagemeister, F.B., Pro, B., McLaughlin, P., Romaguera, J.E., Jones, D., Samuels, B., Samaniego, F., Younes, A., Wang, M., Goy, A., Rodriguez, M.A., Walker, P.L., Arredondo, Y., Tong, A.T. and Fayad, L. (2004). Phase II study of denileukin diftitox for relapsed/refractory B-Cell non-Hodgkin's lymphoma. *J. Clin. Oncol*. **22,** 4095–4102.

Dautin, N., Karimova, G. and Ladant, D. (2002). Bordetella pertussis adenylate cyclase toxin: a versatile screening tool. *Toxicon* **40,** 1383–1387.

Dinges, M.M., Cook, D.R., King, J., Curiel, T.J., Zhang, X.Q. and Harrison, G.S. (1995). HIV-regulated diphtheria toxin A chain gene confers long-term protection against HIV type 1 infection in the human promonocytic cell line U937. *Hum. Gene Ther*. **6,** 1437–1445.

Eklund, J.W. and Kuzel, T.M. (2005). Denileukin diftitox: a concise clinical review. *Expert Rev. Anticancer Ther*. **5,** 33–38.

Ehlich, P. (1956). The relationship existing between chemical constitution, distribution, and pharmacological action. In: *The Collected Papers of Paul Erlich* (ed. F. Himmelweite, *et al.*), pp. 596–618. Vol. I. Pergamon, New York.

Falnes, P.O., Ariansen, S., Sandvig, K. and Olsnes, S. (2000). Requirement for prolonged action in the cytosol for optimal protein synthesis inhibition by diphtheria toxin. *J. Biol. Chem*. **275,** 4363–4368.

Fawcett, J.M., Harrison, S.M. and Orchard, C.H. (1998). A method for reversible permeabilization of isolated rat ventricular myocytes. *Exp. Physiol*. **83,** 293–303.

Fayolle, C., Osickova, A., Osicka, R., Henry, T., Rojas, M.J., Saron, M.F., Sebo, P. and Leclerc, C. (2001). Delivery of multiple epitopes by recombinant detoxified adenylate cyclase of Bordetella pertussis induces protective antiviral immunity. *J. Virol*. **75,** 7330–7338.

Figueiredo, D.M., Hallewell, R.A., Chen, L.L., Fairweather, N.F., Dougan, G., Savitt, J.M., Parks, D.A. and Fishman, P.S. (1997). Delivery of recombinant tetanus-superoxide dismutase proteins to central nervous system neurons by retrograde axonal transport. *Exp. Neurol*. **145,** 546–554.

Finberg, R.W., Wahl, S.M., Allen, J.B., Soman, G., Strom, T.B., Murphy, J.R. and Nichols, J.C. (1991). Selective elimination of HIV-1-infected cells with an interleukin-2 receptor-specific cytotoxin. *Science* **252,** 1703–1705.

Fisher, C.E., Sutherland, J.A., Krause, J.E., Murphy, J.R., Leeman, S.E. and vanderSpek, J.C. (1996). Genetic construction and properties of a diphtheria toxin-related substance P fusion protein: *in vitro* destruction of cells bearing substance P receptors. *Proc. Natl Acad. Sci. USA* **93,** 7341–7345.

Fisher, K.J. and Wilson, J.M. (1997). The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. *Biochem. J*. **321,** 49–58.

Fishman, P.S. and Savitt, J.M. (1989). Transsynaptic transfer of retrogradely transported tetanus protein-peroxidase conjugates. *Exp. Neurol*. **106,** 197–203.

Fishman, P.S., Savitt, J.M. and Farrand, D.A. (1990). Enhanced CNS uptake of systemically administered proteins through conjugation with tetanus C-fragment. *J. Neurol. Sci*. **98,** 311–325.

FitzGerald, D.J., Kreitman, R., Wilson, W., Squires, D. and Pastan, I. (2004). Recombinant immunotoxins for treating cancer. *Int. J. Med. Microbiol*. **293,** 577–582.

Foss, F.M. (2000). DAB(389)IL-2 (ONTAK): a novel fusion toxin therapy for lymphoma. *Clin. Lymphoma* **1,** 110–116.

Francis, J.W., Brown, R.H., Jr., Figueiredo, D., Remington, M.P., Castillo, O., Schwarzschild, M.A., Fishman, P.S., Murphy, J.R. and vanderSpek, J.C. (2000). Enhancement of diphtheria toxin potency by replacement of the receptor-binding domain with tetanus toxin C-fragment: a potential vector for delivering heterologous proteins to neurons. *J. Neurochem*. **74,** 2528–2536.

Frankel, A.E., McCubrey, J.A., Miller, M.S., Delatte, S., Ramage, J., Kiser, M., Kucera, G.L., Alexander, R.L., Beran, M., Tagge, E.P., Kreitman, R.J. and Hogge, D.E. (2000). Diphtheria toxin fused to human interleukin-3 is toxic to blasts from patients with myeloid leukemias. *Leukemia* **14,** 576–585.

Frankel, A.E., Powell, B.L., Hall, P.D., Case, L.D. and Kreitman, R.J. (2002). Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. *Clin. Cancer Res*. **8,** 1004–1013.

Freytag, L.C. and Clements, J.D. (2005). Mucosal adjuvants. *Vaccine* **23,** 1804–1813.

Gottlieb, S.L., Gilleaudeau, P., Johnson, R., Estes, L., Woodworth, T.G., Gottlieb, A.B. and Krueger, J.G. (1995). Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat. Med*. **1,** 442–447.

Gress, J.O., Marquis-Omer, D., Middaugh, C.R. and Sanyal, G. (1994). Evidence for an equilibrium intermediate in the folding-unfolding pathway of a transforming growth factor-alpha-Pseudomonas exotoxin hybrid protein. *Biochemistry* **33,** 2620–2627.

Gu, L.Q., Braha, O., Conlan, S., Cheley, S. and Bayley, H. (1999). Stochastic sensing of organic analytes by a pore-forming protein containing a molecular adapter. *Nature* **398,** 686–690.

Gu, L.Q. and Bayley, H. (2000a). Interaction of the non-covalent molecular adapter, beta-cyclodextrin, with the staphylococcal alpha-hemolysin pore. *Biophys. J.* **79,** 1967–1975.

Gu, L.Q., Dalla Serra, M., Vincent, J.B., Vigh, G., Cheley, S., Braha, O. and Bayley, H. (2000b). Reversal of charge selectivity in transmembrane protein pores by using non-covalent molecular adapters. *Proc. Natl Acad. Sci. USA* **97,** 3959–3964.

Guermonprez, P., Khelef, N., Blouin, E., Rieu, P., Ricciardi-Castagnoli, P., Guiso, N., Ladant, D. and Leclerc, C. (2001). The adenylate cyclase toxin of Bordetella pertussis binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18). *J. Exp. Med.* **193,** 1035–1044.

Haicheur, N., Bismuth, E., Bosset, S., Adotevi, O., Warnier, G., Lacabanne, V., Regnault, A., Desaymard, C., Amigorena, S., Ricciardi-Castagnoli, P., Goud, B., Fridman, W.H., Johannes, L. and Tartour, E. (2000). The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens. *J. Immunol.* **165,** 3301–3308.

Haicheur, N., Benchetrit, F., Amessou, M., Leclerc, C., Falguieres, T., Fayolle, C., Bismuth, E., Fridman, W.H., Johannes, L. and Tartour, E. (2003). The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a Th1-dominant polarization. *Int. Immunol.* **15,** 1161–1171.

Hall, P.D., Virella, G., Willoughby, T., Atchley, D.H., Kreitman, R.J. and Frankel, A.E. (2001). Antibody response to DT-GM, a novel fusion toxin consisting of a truncated diphtheria toxin (DT) linked to human granulocyte-macrophage colony stimulating factor (GM), during a phase I trial of patients with relapsed or refractory acute myeloid leukemia. *Clin. Immunol.* **100,** 191–197.

Harder, T., Scheiffele, P., Verkade, P. and Simons, K. (1998). Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* **141,** 929–942.

Hesketh, P., Caguioa, P., Koh, H., Dewey, H., Facada, A., McCaffrey, R., Parker, K., Nylen, P. and Woodworth, T. (1993). Clinical activity of a cytotoxic fusion protein in the treatment of cutaneous T cell lymphoma. *J. Clin. Oncol.* **11,** 1682–1690.

Ho, V.T., Zahrieh, D., Hochberg, E., Micale, E., Levin, J., Reynolds, C., Steckel, S., Cutler, C., Fisher, D.C., Lee, S.J., Alyea, E.P., Ritz, J., Soiffer, R.J. and Antin, J.H. (2004). Safety and efficacy of denileukin diftitox in patients with steroid-refractory acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Blood* **104,** 1224–1226.

Hotchkiss, C.E., Hall, P.D., Cline, J.M., Willingham, M.C., Kreitman, R.J., Gardin, J., Latimer, A., Ramage, J., Feely, T., DeLatte, S., Tagge, E.P. and Frankel, A.E. (1999). Toxicology and pharmacokinetics of DTGM, a fusion toxin consisting of a truncated diphtheria toxin (DT388) linked to human granulocyte-macrophage colony-stimulating factor, in cynomolgus monkeys. *Toxicol. Appl. Pharmacol.* **158,** 152–160.

Hotz, H.G., Gill, P.S., Masood, R., Hotz, B., Buhr, H.J., Foitzik, T., Hines, O.J. and Reber, H.A. (2002). Specific targeting of tumor vasculature by diphtheria toxin-vascular endothelial growth factor fusion protein reduces angiogenesis and growth of pancreatic cancer. *J. Gastrointest. Surg.* **6,** 159–166.

Hubbard, W.J., Moore, J.K., Contreras, J.L., Smyth, C.A., Chen, Z.W., Lobashevsky, A.L., Nagata, K., Neville, D.M., Jr. and Thomas, J.M. (2001). Phenotypic and functional analysis of T cell recovery after anti-CD3 immunotoxin treatment for tolerance induction in rhesus macaques. *Hum. Immunol.* **62,** 479–487.

Jankovic, J. (2004). Botulinum toxin in clinical practice. *J. Neurol. Neurosurg. Psychiatry* **75,** 951–957.

Jansson, B., Uhlen, M. and Nygren, P.A. (1998). All individual domains of staphylococcal protein A show Fab binding. *FEMS Immunol. Med. Microbiol.* **20,** 69–78.

Jedema, I., Barge, R.M., Frankel, A.E., Willemze, R. and Falkenburg, J.H. (2004). Acute myeloid leukemia cells in G0 phase of the cell cycle that are unresponsive to conventional chemotherapy are sensitive to treatment with granulocyte-macrophage, colony-stimulating factor/diphtheria toxin fusion proteins. *Exp. Hematol.* **32,** 188–194.

Kissa, K., Mordelet, E., Soudais, C., Kremer, E.J., Demeneix, B.A., Brulet, P. and Coen, L. (2002). *In vivo* neuronal tracing with GFP-TTC gene delivery. *Mol. Cell. Neurosci.* **20,** 627–637.

Kiyokawa, T., Shirono, K., Hattori, T., Nishimura, H., Yamaguchi, K., Nichols, J.C., Strom, T.B., Murphy, J.R. and Takatsuki, K. (1989). Cytotoxicity of interleukin 2-toxin toward lymphocytes from patients with adult T cell leukemia. *Cancer Res.* **49,** 4042–4046.

Klingenberg, O. and Olsnes, S. (1996). Ability of methotrexate to inhibit translocation to the cytosol of dihydrofolate reductase fused to diphtheria toxin. *Biochem. J.* **313,** 647–653.

Koriazova, L.K. and Montal, M. (2003). Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat. Struct. Biol.* **10,** 13–18.

Kreitman, R.J. and Pastan, I. (1995). Targeting Pseudomonas exotoxin to hematologic malignancies. *Semin. Cancer Biol.* **6,** 297–306.

Kreitman, R.J., Wilson, W.H., Bergeron, K., Raggio, M., Stetler-Stevenson, M., FitzGerald, D.J. and Pastan, I. (2001). Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant, hairy-cell leukemia. *N. Engl. J. Med.* **345,** 241–247.

Kuan, C.T., Pai, L.H. and Pastan, I. (1995). Immunotoxins containing Pseudomonas exotoxin that target LeY damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome. *Clin. Cancer Res.* **1,** 1589–1594.

Lakkis, F., Steele, A., Pacheco-Silva, A., Rubin-Kelley, V., Strom, T.B. and Murphy, J.R. (1991). Interleukin 4 receptor targeted cytotoxicity: genetic construction and *in vivo* immunosuppressive activity of a diphtheria toxin-related murine interleukin 4 fusion protein. *Eur. J. Immunol.* **21,** 2253–2258.

Landgraf, R., Pegram, M., Slamon, D.J. and Eisenberg, D. (1998). Cytotoxicity and specificity of directed toxins composed of diphtheria toxin and the EGF-like domain of heregulin beta1. *Biochemistry* **37,** 3220–3228.

Lee, B.K., Yoo, J.E., Jang, Y.S., Kim, J.Y., Hong, C.S. and Ro, J.Y. (2004). Allergen-specific immunosuppression by ovalbumin fused with diphtheria toxin in mice sensitized with albumins of different origin. *Clin. Exp. Allergy* **34,** 1642–1648.

Lee, R.S., Tartour, E., van der Bruggen, P., Vantomme, V., Joyeux, I., Goud, B., Fridman, W.H. and Johannes, L. (1998). Major histocompatibility complex class I presentation of exogenous soluble tumor antigen fused to the B-fragment of Shiga toxin. *Eur. J. Immunol.* **28,** 2726–2737.

LeMaistre, C.F., Meneghetti, C., Howes, L. and Osborne, C.K. (1994). Targeting the EGF receptor in breast cancer treatment. *Breast Cancer Res. Treat.* **32,** 97–103.

LeMaistre, C.F., Saleh, M.N., Kuzel, T.M., Foss, F., Platanias, L.C., Schwartz, G., Ratain, M., Rook, A., Freytes, C.O., Craig, F., Reuben, J. and Nichols, J.C. (1998). Phase I trial of a ligand fusion-protein (DAB389IL-2) in lymphomas expressing the receptor for interleukin-2. *Blood* **91,** 399–405.

LeMaistre, C.F. (2000). DAB(389)IL-2 (denileukin diftitox, ONTAK): other potential applications. *Clin. Lymphoma 1 Suppl* **1,** 37–40.

Lemichez, E., Bomsel, M., Devilliers, G., vanderSpek, J., Murphy, J.R., Lukianov, E.V., Olsnes, S. and Boquet, P. (1997). Membrane

translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. *Mol. Microbiol*. **23,** 445–457.

Li, C., Hall, W.A., Jin, N., Todhunter, D.A., Panoskaltsis-Mortari, A. and Vallera, D.A. (2002). Targeting glioblastoma multiforme with an IL-13/diphtheria toxin fusion protein *in vitro* and *in vivo* in nude mice. *Protein Eng*. **15,** 419–427.

Liger, D., vanderSpek, J.C., Gaillard, C., Cansier, C., Murphy, J.R., Leboulch, P. and Gillet, D. (1997). Characterization and receptor specific toxicity of two diphtheria toxin-related interleukin-3 fusion proteins DAB389-mIL-3 and DAB389-(Gly4Ser)2-mIL-3. *FEBS Lett*. **406,** 157–161.

Liger, D., Nizard, P., Gaillard, C., vanderSpek, J.C., Murphy, J.R., Pitard, B. and Gillet, D. (1998). The diphtheria toxin transmembrane domain as a pH sensitive membrane anchor for human interleukin-2 and murine interleukin-3. *Protein Eng*. **11,** 1111–1120.

Liu, T.F., Hall, P.D., Cohen, K.A., Willingham, M.C., Cai, J., Thorburn, A. and Frankel, A.E. (2005). Interstitial diphtheria toxin-epidermal growth factor fusion protein therapy produces regressions of subcutaneous human glioblastoma multiformed tumors in athymic nude mice. *Clin. Cancer Res*. **11,** 329–334.

Ljungberg, U.K., Jansson, B., Niss, U., Nilsson, R., Sandberg, B.E. and Nilsson, B. (1993). The interaction between different domains of staphylococcal protein A and human polyclonal IgG, IgA, IgM, and F(ab')2: separation of affinity from specificity. *Mol. Immunol*. **30,** 1279–1285.

Lobeck, K., Drevet, P., Leonetti, M., Fromen-Romano, C., Ducancel, F., Lajeunesse, E., Lemaire, C. and Menez, A. (1998). Towards a recombinant vaccine against diphtheria toxin. *Infect. Immun*. **66,** 418–423.

Lorberboum-Galski, H., FitzGerald, D., Chaudhary, V., Adhya, S. and Pastan, I. (1988). Cytotoxic activity of an interleukin 2-Pseudomonas exotoxin chimeric protein produced in Escherichia coli. *Proc. Natl Acad. Sci. USA* **85,** 1922–1926.

Ma, S., Hu, H., Thompson, J., Stavrou, S., Scharff, J. and Neville, D.M., Jr. (1997). Genetic construction and characterization of an anti-monkey CD3 single-chain immunotoxin with a truncated diphtheria toxin. *Bioconjug. Chem*. **8,** 695–701.

Madshus, I.H., Olsnes, S. and Stenmark, H. (1992). Membrane translocation of diphtheria toxin carrying passenger protein domains. *Infect. Immun*. **60,** 3296–3302.

Martin-Serrano, J., Folgueira, L., Lain de Lera, T., Pedraza, M.A., Lemichez, E., Sanchez-Palomino, S., Noriega, A.R., Boquet, P. and Alcami, J. (1998). *In vitro* selective elimination of HIV-infected cells from peripheral blood in AIDS patients by the immunotoxin DAB389CD4. *AIDS* **12,** 859–863.

Marvaud, J.C., Stiles, B.G., Chenal, A., Gillet, D., Gibert, M., Smith, L.A. and Popoff, M.R. (2002). Clostridium perfringens iota toxin. Mapping of the Ia domain involved in docking with Ib and cellular internalization. *J. Biol. Chem*. **277,** 43659–43666.

Masood, R., Lunardi-Iskandar, Y., Jean, L.F., Murphy, J.R., Waters, C., Gallo, R.C. and Gill, P. (1994). Inhibition of AIDS-associated Kaposi's sarcoma cell growth by DAB389-interleukin 6. *AIDS Res. Hum. Retroviruses* **10,** 969–975.

Masood, R., Kundra, A., Zhu, S., Xia, G., Scalia, P., Smith, D.L. and Gill, P.S. (2003). Malignant mesothelioma growth inhibition by agents that target the VEGF and VEGF-C autocrine loops. *Int. J. Cancer* **104,** 603–610.

Menetrey, J., Gillet, D. and Menez, A. (2005). Structural features common to intracellularly acting toxins from bacteria. *Toxicon* **45,** 129–137.

Mere, J., Morlon-Guyot, J., Bonhoure, A., Chiche, L. and Beaumelle, B. (2005). Acid-triggered membrane insertion of Pseudomonas exotoxin A involves an original mechanism based on pH-regulated tryptophan exposure. *J. Biol. Chem. Online*, Epub ahead of print.

Milne, J.C., Blanke, S.R., Hanna, P.C. and Collier, R.J. (1995). Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus. *Mol. Microbiol*. **15,** 661–666.

Mishra, G., Liu, T.F. and Frankel, A.E. (2003). Recombinant toxin DAB389EGF is cytotoxic to human pancreatic cancer cells. *Expert Opin Biol Ther* **3,** 1173–1180.

Mitamura, T., Higashiyama, S., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995). Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. *J. Biol. Chem*. **270,** 1015–1019.

Moreland, L.W., Sewell, K.L., Trentham, D.E., Bucy, R.P., Sullivan, W.F., Schrohenloher, R.E., Shmerling, R.H., Parker, K.C., Swartz, W.G. and Woodworth, T.G. *et al*. (1995). Interleukin-2 diphtheria fusion protein (DAB486IL-2) in refractory rheumatoid arthritis. A double-blind, placebo-controlled trial with open-label extension. *Arthritis Rheum*. **38,** 1177–1186.

Murphy, J.R., Bishai, W., Borowski, M., Miyanohara, A., Boyd, J. and Nagle, S. (1986). Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion protein. *Proc. Natl Acad. Sci. USA* **83,** 8258–8262.

Murphy, J.R. and vanderSpek, J.C. (1995). Targeting diphtheria toxin to growth factor receptors. *Semin. Cancer Biol*. **6,** 259–267.

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69,** 1051–1061.

Nizard, P., Liger, D., Gaillard, C. and Gillet, D. (1998). Anchoring antibodies to membranes using a diphtheria toxin T domain-ZZ fusion protein as a pH sensitive membrane anchor. *FEBS Lett*. **433,** 83–88.

Nizard, P., Chenal, A., Beaumelle, B., Fourcade, A. and Gillet, D. (2001). Prolonged display or rapid internalization of the IgG-binding protein ZZ anchored to the surface of cells using the diphtheria toxin T domain. *Protein Eng*. **14,** 439–446.

Nizard, P., Gross, D.A., Chenal, A., Beaumelle, B., Kosmatopoulos, K. and Gillet, D. (2002). Novel cancer vaccines prepared by anchoring cytokines to tumor cells avoiding gene transfection. In: *Clinical Diagnostic Systems: Technologies and Instrumentation* (ed. G.E. Cohn), pp. 118–125. Vol. 4625. Proceedings of SPIES, Washington

Nizard, P., Gross, D.A., Babon, A., Chenal, A., Beaumelle, B., Kosmatopoulos, K. and Gillet, D. (2003). Anchoring cytokines to tumor cells for the preparation of anticancer vaccines without gene transfection in mice. *J. Immunother*. **26,** 63–71.

Ogata, M., Chaudhary, V.K., FitzGerald, D.J. and Pastan, I. (1989). Cytotoxic activity of a recombinant fusion protein between interleukin 4 and Pseudomonas exotoxin. *Proc. Natl. Acad. Sci. USA* **86,** 4215–4219.

Olson, R., Nariya, H., Yokota, K., Kamio, Y. and Gouaux, E. (1999). Crystal structure of staphylococcal LukF delineates conformational changes accompanying formation of a transmembrane channel. *Nat. Struct. Biol*. **6,** 134–140.

Olson, T.A., Mohanraj, D., Roy, S. and Ramakrishnan, S. (1997). Targeting the tumor vasculature: inhibition of tumor growth by a vascular endothelial growth factor-toxin conjugate. *Int. J. Cancer* **73,** 865–870.

Padungchaichot, P., Wong, J.Y., Natoli, A.L., Massalas, J.S., Finkelstein, D.I., Lawrence, A.L. and Drago, J. (2000). Early direct and transneuronal effects in mice with targeted expression of a

toxin gene to D1 dopamine receptor neurons. *Neuroscience* **95,** 1025–1033.

Paliwal, R. and London, E. (1996). Comparison of the conformation, hydrophobicity, and model membrane interactions of diphtheria toxin to those of formaldehyde-treated toxin (diphtheria toxoid): formaldehyde stabilization of the native conformation inhibits changes that allow membrane insertion. *Biochemistry* **35,** 2374–2379.

Panchal, R.G., Cusack, E., Cheley, S. and Bayley, H. (1996). Tumor protease-activated, pore-forming toxins from a combinatorial library. *Nat. Biotechnol*. **14,** 852–856.

Parker, M.W. and Feil, S.C. (2005). Pore-forming protein toxins: from structure to function. *Prog. Biophys. Mol. Biol*. **88,** 91–142.

Pastan, I. (2003). Immunotoxins containing Pseudomonas exotoxin A: a short history. *Cancer Immunol. Immunother*. **52,** 338–341.

Pitcher, C., Roberts, L., Fawell, S., Zdanovsky, A.G., FitzGerald, D.J. and Lord, J.M. (1995). Generation of a potent chimeric toxin by replacement of domain III of Pseudomonas exotoxin with ricin A chain KDEL. *Bioconjug. Chem*. **6,** 624–629.

Ramage, J.G., Vallera, D.A., Black, J.H., Aplan, P.D., Kees, U.R. and Frankel, A.E. (2003). The diphtheria toxin/urokinase fusion protein (DTAT) is selectively toxic to CD87 expressing leukemic cells. *Leuk. Res*. **27,** 79–84.

Richard, J.F., Petit, L., Gibert, M., Marvaud, J.C., Bouchaud, C. and Popoff, M.R. (1999). Bacterial toxins modifying the actin cytoskeleton. *Int. Microbiol*. **2,** 185–194.

Rosenkranz, A.A., Lunin, V.G., Gulak, P.V., Sergienko, O.V., Shumiantseva, M.A., Voronina, O.L., Gilyazova, D.G., John, A.P., Kofner, A.A., Mironov, A.F., Jans, D.A. and Sobolev, A.S. (2003). Recombinant modular transporters for cell-specific nuclear delivery of locally acting drugs enhance photosensitizer activity. *FASEB J*. **17,** 1121–1123.

Russo, M.J., Bayley, H. and Toner, M. (1997). Reversible permeabilization of plasma membranes with an engineered switchable pore. *Nat. Biotechnol*. **15,** 278–282.

Saito, M., Iwawaki, T., Taya, C., Yonekawa, H., Noda, M., Inui, Y., Mekada, E., Kimata, Y., Tsuru, A. and Kohno, K. (2001). Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol*. **19,** 746–750.

Saron, M.F., Fayolle, C., Sebo, P., Ladant, D., Ullmann, A. and Leclerc, C. (1997). Anti-viral protection conferred by recombinant adenylate cyclase toxins from Bordetella pertussis carrying a CD8+ T cell epitope from lymphocytic choriomeningitis virus. *Proc. Natl Acad. Sci. USA* **94,** 3314–3319.

Schmidt, W., Schweighoffer, T., Herbst, E., Maass, G., Berger, M., Schilcher, F., Schaffner, G. and Birnstiel, M.L. (1995). Cancer vaccines: the interleukin 2 dosage effect. *Proc. Natl. Acad. Sci. USA* **92,** 4711–4714.

Sharma, M., Khanna, H., Arora, N. and Singh, Y. (2000). Anthrax toxin-mediated delivery of cholera toxin-A subunit into the cytosol of mammalian cells. *Biotechnol. Appl. Biochem*. **32**(Pt 1), 69–72.

Siegall, C.B., Chaudhary, V.K., FitzGerald, D.J. and Pastan, I. (1988). Cytotoxic activity of an interleukin 6-Pseudomonas exotoxin fusion protein on human myeloma cells. *Proc. Natl. Acad. Sci. USA* **85,** 9738–9742.

Siegall, C.B., Xu, Y.H., Chaudhary, V.K., Adhya, S., Fitzgerald, D. and Pastan, I. (1989). Cytotoxic activities of a fusion protein comprised of TGF alpha and Pseudomonas exotoxin. *FASEB J*. **3,** 2647–2652.

Siegall, C.B., Liggitt, D., Chace, D., Mixan, B., Sugai, J., Davidson, T. and Steinitz, M. (1997). Characterization of vascular leak syndrome induced by the toxin component of Pseudomonas exotoxin-based immunotoxins and its potential inhibition with non-steroidal anti-inflammatory drugs. *Clin. Cancer Res*. **3,** 339–345.

Smith, D.C., Lord, J.M., Roberts, L.M., Tartour, E. and Johannes, L. (2002a). First class ticket to class I: protein toxins as pathfinders for antigen presentation. *Traffic* **3,** 697–704.

Smith, D.C., Gallimore, A., Jones, E., Roberts, B., Lord, J.M., Deeks, E., Cerundolo, V. and Roberts, L.M. (2002b). Exogenous peptides delivered by ricin require processing by signal peptidase for transporter associated with antigen processing-independent MHC class I-restricted presentation. *J. Immunol*. **169,** 99–107.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J.E. (1996). Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* **274,** 1859–1866.

Stenmark, H., Moskaug, J.O., Madshus, I.H., Sandvig, K. and Olsnes, S. (1991). Peptides fused to the amino-terminal end of diphtheria toxin are translocated to the cytosol. *J. Cell Biol*. **113,** 1025–1032.

Sundan, A., Olsnes, S., Sandvig, K. and Pihl, A. (1982). Preparation and properties of chimeric toxins prepared from the constituent polypeptides of diphtheria toxin and ricin. Evidence for entry of ricin A-chain via the diphtheria toxin pathway. *J. Biol. Chem*. **257,** 9733–9739.

Sweeney, E.B., Foss, F.M., Murphy, J.R. and vanderSpek, J.C. (1998). Interleukin 7 (IL-7) receptor-specific cell killing by DAB389 IL-7: a novel agent for the elimination of IL-7 receptor positive cells. *Bioconjug. Chem*. **9,** 201–207.

Tatro, J.B., Wen, Z., Entwistle, M.L., Atkins, M.B., Smith, T.J., Reichlin, S. and Murphy, J.R. (1992). Interaction of an alpha-melanocyte-stimulating, hormone-diphtheria toxin fusion protein with melanotropin receptors in human melanoma metastases. *Cancer Res*. **52,** 2545–2548.

Tavallaie, M., Chenal, A., Gillet, D., Pereira, Y., Manich, M., Gibert, M., Raffestin, S., Popoff, M.R. and Marvaud, J.C. (2004). Interaction between the two subdomains of the C-terminal part of the botulinum neurotoxin A is essential for the generation of protective antibodies. *FEBS Lett*. **572,** 299–306.

Todhunter, D.A., Hall, W.A., Rustamzadeh, E., Shu, Y., Doumbia, S.O. and Vallera, D.A. (2004). A bispecific immunotoxin (DTAT13) targeting human IL-13 receptor (IL-13R) and urokinase-type plasminogen activator receptor (uPAR) in a mouse xenograft model. *Protein Eng. Des. Sel*. **17,** 157–164.

Trowbridge, I.S. and Domingo, D.L. (1981). Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumor cells. *Nature* **294,** 171–173.

Tsuneoka, M., Nakayama, K., Hatsuzawa, K., Komada, M., Kitamura, N. and Mekada, E. (1993). Evidence for involvement of furin in cleavage and activation of diphtheria toxin. *J. Biol. Chem*. **268,** 26461–26465.

Tuo, H.Z., Wang, J.W., Wang, D.X., Li, J.M., Ouyang, J. and Hong, T. (2004). Construction and expression of the fusion protein DT389-hIL-13 and its cytotoxicity to glioma cell lines. *Zhonghua Yi Xue Za Zhi* **84,** 1024–1028.

Uherek, C., Fominaya, J. and Wels, W. (1998). A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *J. Biol. Chem*. **273,** 8835–8841.

Vallera, D.A., Panoskaltsis-Mortari, A. and Blazar, B.R. (1997). Renal dysfunction accounts for the dose-limiting toxicity of DT390antiCD3sFv, a potential new recombinant anti-GVHD immunotoxin. *Protein Eng*. **10,** 1071–1076.

Vallera, D.A., Seo, S.Y., Panoskaltsis-Mortari, A., Griffin, J.D. and Blazar, B.R. (1999). Targeting myeloid leukemia with a DT(390)-mIL-3 fusion immunotoxin: *ex vivo* and *in vivo* studies in mice. *Protein Eng*. **12,** 779–785.

Vallera, D.A., Todhunter, D., Kuroki, D.W., Shu, Y., Sicheneder, A., Panoskaltsis-Mortari, A., Vallera, V.D. and Chen, H. (2005).

Molecular modification of a recombinant, bivalent anti-human CD3 immunotoxin (Bic3) results in reduced *in vivo* toxicity in mice. *Leuk. Res*. **29,** 331–341.

vanderSpek, J.C., Sutherland, J., Sampson, E. and Murphy, J.R. (1995). Genetic construction and characterization of the diphtheria toxin-related interleukin 15 fusion protein DAB389 sIL-15. *Protein Eng*. **8,** 1317–1321.

vanderSpek, J.C., Sutherland, J.A., Zeng, H., Battey, J.F., Jensen, R.T. and Murphy, J.R. (1997). Inhibition of protein synthesis in small cell lung cancer cells induced by the diphtheria toxin-related fusion protein DAB389 GRP. *Cancer Res*. **57,** 290–294.

Walev, I., Bhakdi, S.C., Hofmann, F., Djonder, N., Valeva, A., Aktories, K. and Bhakdi, S. (2001). Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl Acad. Sci. USA* **98,** 3185–3190.

Walker, B. and Bayley, H. (1994). A pore-forming protein with a protease-activated trigger. *Protein Eng*. **7,** 91–97.

Walker, B., Kasianowicz, J., Krishnasastry, M. and Bayley, H. (1994). A pore-forming protein with a metal-actuated switch. *Protein Eng*. **7,** 655–662.

Waters, C.A., Schimke, P.A., Snider, C.E., Itoh, K., Smith, K.A., Nichols, J.C., Strom, T.B. and Murphy, J.R. (1990). Interleukin 2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells. *Eur. J. Immunol*. **20,** 785–791.

Wiedlocha, A., Madshus, I.H., Mach, H., Middaugh, C.R. and Olsnes, S. (1992). Tight folding of acidic fibroblast growth factor prevents its translocation to the cytosol with diphtheria toxin as vector. *EMBO J*. **11,** 4835–4842.

Wild, R., Yokoyama, Y., Dings, R.P. and Ramakrishnan, S. (2004). VEGF-DT385 toxin conjugate inhibits mammary adenocarcinoma development in a transgenic mouse model of spontaneous tumorigenesis. *Breast Cancer Res. Treat*. **85,** 161–171.

Williams, D.P., Parker, K., Bacha, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T.B. and Murphy, J.R. (1987). Diphtheria toxin receptor-binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. *Protein Eng*. **1,** 493–498.

Wong, J.Y., Padungchaichot, P., Massalas, J.S. and Drago, J. (2000). Late direct and transneuronal effects in mice with targeted expression of a toxin gene to D1 dopamine receptor neurons. *Neuroscience* **95,** 1035–1041.

Woodworth, T.G. and Nichols, J.C. (1993). Recombinant fusion toxins—a new class of targeted biologic therapeutics. *Cancer Treat. Res*. **68,** 145–160.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15,** 245–250.

CHAPTER

# 61

# Engineered bacterial toxin vaccines and adjuvants

*Jan Holmgren and Ann-Mari Svennerholm*

## INTRODUCTION

There has been remarkable progress in the understanding of the molecular biology of bacterial toxins in recent years, linking the fairly detailed knowledge of the structure-function relationship of several bacterial toxins with an advanced understanding of toxin genes and the regulation of their expression as described in this volume. This has resulted in completely new possibilities to prepare detoxified yet immunologically active toxin derivates. In combination with the similarly rapid progress in biotechnology, dramatic improvements in the potentials for preparing rationally designed vaccine immunogens based on recombinantly produced, genetically engineered, detoxified toxins or non-toxic subunits now are at hand (Holmgren and Svennerholm, 1998; Del Giudice and Rappuoli, 1999).

Recently, engineered detoxified toxins or non-toxic subunits have also attracted much interest as vaccine carrier proteins, adjuvants, and/or immunomodulators, in some cases for parenteral use but especially for mucosal administration (Holmgren and Lycke, 1993; Lycke, 1997; Gagliardi *et al*., 2002). Mucosal immunization has for good reasons attracted much interest recently, mainly as a means to induce protective immunity against the many important infections, which either take place at and affect mucosal surfaces or have a mucosal surface as their portal of entry (Holmgren and Czerkinsky, 2005; Holmgren and Svennerholm, 2004). Much less well appreciated than vaccination against infections, mucosal immunization may also be used to induce peripheral tolerance to prevent or suppress the development of harmful immune responses to foreign proteins derived from ingested food or from commensal microorganisms, in case these antigens would reach the body interior in an undegraded, immunogenic form (Holmgren and Czerkinsky, 2005; Wu and Weiner, 2003). For both types of applications, it has been found that bacterial toxin derivatives can substantially influence the outcome in ways that give hope for further development toward practical medical use. Likewise, engineered bacterial toxin proteins have found a useful place as carrier proteins for various capsular polysaccharides in the development of conjugate vaccines against important infections with encapsulated bacteria (Vadheim *et al*., 1994; Lieberman *et al*., 1996). Yet a further potential area for use of engineered, and potentially even native, bacterial toxins is as adjuvants for dendritic cell vaccination, which is rapidly becoming a promising treatment complement in the clinical management of many cancer forms (George-Chandy *et al*., 2001; Gagliardi *et al*., 2002; Eriksson *et al*, 2004).

In this chapter, we will briefly describe the properties and clinical potential of various types of genetically engineered bacterial toxins and subunits in these different areas of vaccination, adjuvanting, and immunomodulation.

## CHOLERA TOXIN AND CHOLERA VACCINE DEVELOPMENT

Cholera toxin (CT) was the first toxin that was described in its detailed molecular structure and function, as well as in its complex immunological properties, both

as a protective immunogen and as a potent mucosal adjuvant and immunomodulator (Holmgren, 1981). This included the identification of the AB5 structure with different subunits being responsible for the cell, binding and the toxic activities; the identification of the GM1 ganglioside as the specific cellular receptor for the toxin; the clarification of the pathogenic toxic-enzymatic action of the toxin on the intestinal target cells (ADP-ribosylation of the Gs protein leading to adenylate cyclase activation and increased cyclic AMP formation); and the connection between these effects and the excessive salt and fluid losses characteristic of the cholera disease (Holmgren, 1981; Guerrant, 1985). It also included the identification of the completely non-toxic B subunit pentamer (CTB) rather than the toxic-active A subunit as the target for neutralizing antibodies, thus pointing to CTB as a rational protein to be used as a mucosal vaccine immunogen for inducing effective CT-blocking intestinal immunity that could protect against cholera disease (Holmgren and Svennerholm, 1998; Holmgren and Bergqvist, 2004). This notion was further strengthened by findings in animals showing that immunization with highly purified CTB gave rise to toxin-neutralizing antibodies, which could fully protect against disease not only after toxin challenge but also after challenge with live *V. cholerae* O1 bacteria in the gut (Holmgren *et al*., 1977). Furthermore, CTB has been found to be particularly well suited as an oral immunogen, because of its stability in the intestinal milieu and its high-affinity binding to the intestinal epithelium, including the M-cells of the Peyer's patches, properties that are very important for stimulating mucosal immunity and local immunological memory (Neutra and Kraehenbuhl, 1993).

Based on this knowledge and further work that showed also that antibacterial intestinal cholera immunity is important and that indeed the antibacterial and the antitoxic immune mechanisms cooperate synergistically in protecting against cholera infection and disease (Svennerholm and Holmgren, 1976), the oral CTB-whole cell cholera vaccine was developed (Holmgren *et al*, 1977). This oral cholera vaccine, which is now registered (Dukoral®) in more than 50 countries worldwide, thus represents one of rather few mucosal vaccines available for human use.

The first-generation oral cholera vaccine contained CTB purified from CT produced in a fermentor by the wild-type strain 569B (Tayot *et al*., 1981). This made the preparation of this extensively purified component laborious and relatively expensive. These drawbacks were overcome by the development of an efficient recombinant overexpression system for the large-scale production of CTB (Sanchez and Holmgren, 1989), systems that have since then been further improved upon (Lebens *et al.*, 1993). These latter procedures now yield well over one gram of highly purified CTB per liter of fermentor culture even in 1000-liter fermentations, which is approximately 100-fold better than with the early non-recombinant production and isolation of CTB. Since there is no CTA subunit gene present in the production strain used, no active toxin can be formed, which makes the purification processes further efficient and cheaper (Sanchez and Holmgren, 1989). No differences have been found in the function of recombinantly produced CTB and toxin-derived CTB as a vaccine component. Immunological and functional studies, including immunogenicity studies in both animals and humans, have not been able to demonstrate any differences in the two molecules or in the immune responses they evoke (Jertborn *et al*., 1992). Moreover, the results from an efficacy field trial in Peru with vaccine containing the recombinantly produced CTB (Sanchez *et al*., 1994) and most recently also those from a large effectiveness study in Mozambique (Lucas *et al.*, 2005) were fully consistent with the high efficacy results obtained in Bangladesh with first-generation vaccine containing native CTB (Clemens *et al*., 1988a).

Many studies have shown that the CTB-WC vaccine is safe and immunogenic in both children and adults in cholera-endemic as well as non-endemic countries, stimulating gut mucosal immune responses to the CTB and WC vaccine components (Holmgren and Svennerholm, 1998; Holmgren and Bergqvist, 2004). A high-level short-term and a good long-term protective efficacy of the vaccine has been demonstrated, being 85% for the first six months after vaccination in both endemic and non-endemic populations (Clemens *et al.*, 1986, Sanchez *et al.,* 1994), and remaining at or above 60% for at least two years in vaccinated adults and in children above age five years (Clemens *et al*., 1988a; Clemens *et al*., 1990). In children below age 5, the short-term efficacy is very high (100% for the first six months when tested in a field trial in Bangladesh), but of shorter duration than in older children and adults (Table 61.1).

Protection by the oral CTB-WC cholera vaccine is mediated by locally produced IgA antibacterial and antitoxic antibodies blocking bacterial colonization and cholera toxin action, respectively; for longer-term protection, mucosal memory for rapid induction of mainly anticolonization IgA antibodies is important since these antibodies can then block bacterial colonization and multiplication at the intestinal surface before the infection has progressed to cause disease (Holmgren and Svennerholm, 1998). The antitoxic immunity appears to be particularly important for high-level, early protection in previously immunologically, relatively naïve vaccines, such as represented by young children in cholera-endemic countries, or all

**TABLE 61.1** Evidence of high short-term protective antitoxic protective efficacy against cholera, especially in young children, by the CTB component of the B subunit (CTB)-killed whole cell (WC) oral cholera vaccine

| | | Protective efficacy % | | |
|---|---|---|---|---|
| **Field trial** | **Vaccine** | **All ages** | **Children 2–5 y** | **Adults and children >5 y** |
| Bangladesh | CTB-WC vaccine | | | |
| 6 months | | 85 | 100 | 76 |
| 2 years | | 60 | 41 | 71 |
| Peru | rCTB-WC vaccine | 85 | Not included | 85 |
| Mozambique | rCTB-WC vaccine | 78 | 82 | 67 |
| Bangladesh | | | | |
| 6 months | WC vaccine | 58 | 35 | 71 |
| 2 years | | 56 | 27 | 70 |
| Vietnam | WC vaccine | 66 | 68 | 66 |

ages when outbreaks first occur in previously unaffected countries, or in travelers to endemic countries. This is illustrated by the data in Table 61.1, showing significantly higher short-term efficacy, especially in young children in Bangladesh, of the CTB-WC vaccine compared to the WC vaccine without CTB in a side-by-side comparison in the same trial (Clemens *et al*, 1986). The same notion is also supported by the much higher efficacy in previously cholera-spared adult Peruvians, and especially in children in a recent effectiveness study in Mozambique using rCTB-WC vaccine, than in both Bangladeshi and Vietnamese people vaccinated with only WC vaccine (Table 61.1). For long-term protection in endemic countries, antibacterial immunity, largely based on immunologic memory stimulation, appears to be most important.

The CTB-WC cholera vaccine also offers significant short-term cross-protection (through the CTB component) against diarrhea caused by enterotoxigenic *E. coli* (ETEC) producing the heat-labile enterotoxin (LT), which cross-reacts immunologically with CT (Table 61.2; Clemens *et al*., 1988b; Peltola *et al*., 1991; Scerpella *et al*, 1995). Currently there is no other vaccine available against ETEC. The CTB-WC cholera vaccine has also been shown to give rise to substantial herd immunity and has been found to substantially reduce overall diarrhea morbidity in areas where cholera and ETEC-diarrheas are common *et al*. A large effectiveness study to assess the role of the vaccine in public health control programs against cholera in Africa has recently been undertaken. Results show that protection even under these conditions and in a population where about 30% may be HIV infected is similar to the efficacy demonstrated in the previously cited phase 3 efficacy trials, being about 80% against cholera as presented to the local treatment centers and nearly 90% against more severe cholera disease associated with dehydration (Lucas *et al*., 2005).

**TABLE 61.2** Protection by oral CTB-WC cholera vaccine against ETEC producing LT or LT/ST in different studies

| **Study** | **Protective efficacy** | **Reference** |
|---|---|---|
| Field trial in Bangladesh | 67% | Clemens *et al*. (1988b) |
| Finnish travelers to Morocco | 60% | Peltola *et al*. (1991) |
| U.S. students to Mexico | 50% | Scerpella *et al*. (1995) |

A second type of oral cholera vaccine, based on a live attenuated *V. cholerae* strain deleted in its cholera toxin gene from the ability to make the toxic-active A subunit, is also registered in some countries for use in travelers (Levine and Kaper, 1995; Levine and Tacket, 1995). However, different from the CTB-WC vaccine, the live, attenuated cholera vaccine failed to generate significant protection when tested in a large field trial in Indonesia, and thus is not recommended by WHO for use in cholera-endemic countries (Richie *et al*., 2000; WHO, 2001). Although this vaccine strain may produce tiny amounts of B subunit, the antitoxic immune responses are meager and protection in volunteers appears to be exclusively mediated by mucosal antibacterial immunity. The same appears to hold true also for newer, live, attenuated oral cholera vaccine candidates, wherein the cholera toxin gene has been modified to render the strain non-toxic, such as the Peru-15 vaccine strain (Kenner *et al*., 1995), which currently is in phase 2 clinical testing (Cohen *et al*., 2002).

## TOXIN-BASED VACCINES AGAINST ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC)

Enterotoxigenic *Escherichia coli* (ETEC) cause disease by colonizing the small intestine and elaborating LT or heat-stable enterotoxin (ST) or both toxins (Sack, 1980). The resulting illness usually lasts from 3–5 days and ranges from mild diarrhea without dehydration to severe cholera-like disease (Svennerholm and Steele, 2004). Though the illness is usually mild, it is occasionally cholera-like in its severity and causes an estimated 300–500,000 deaths per year, mostly in young children. This high incidence in developing countries and in travelers to these areas makes ETEC an important target

for immunoprophylaxis (Black, 1990, 1993). In regions of the world where ETEC is highly endemic, there is a decline in ETEC diarrhea incidence with age. This, together with the findings that the oral CTB-WC cholera vaccine induced highly significant protection against LT-producing ETEC, strongly supports the notion that an effective enterotoxin-based ETEC vaccine is achievable.

The critical virulence properties that contribute to the pathogenesis of ETEC diarrhea are the ability of bacteria to adhere to the mucosa of the proximal small intestine by means of specialized, usually fimbrial attachment proteins (so-called colonization factors, CFs) and to elaborate LT and/or ST enterotoxins (Gaastra and Svennerholm, 1996). Protective immunity against ETEC appears to be mediated by mainly secretory IgA antibodies directed against LT, CFs, and other surface antigens; ST, which is a low-molecular weight peptide, does not elicit neutralizing antibodies following natural infection (Holmgren and Svennerholm, 1998).

To provide broad spectrum protection, an ETEC vaccine should contain an appropriate "toxoid" together with fimbrial antigens representative of the most prevalent ETEC pathogens. In view of the fact that ST is not immunogenic in its own right, it is especially important that the CF antigens cover a broad range of ETEC strains producing ST. The most prevalent CF antigens on ST only or ST/LT producing human ETEC isolates are CFA/I, CS1-CS3 (CFA/II), and CS4-CS6 (CFA/IV). Thus, a multivalent ETEC vaccine containing an LT toxoid together with these CFs is estimated to be able to protect against about 80% of ETEC strains worldwide (Svennerholm and Savarino, 2004).

Different strategies have been taken to deliver ETEC toxin antigens and fimbriae to the human immune system to elicit protective immune responses in the form of inactivated or live candidate vaccines. An attractive approach seemed to be to prepare killed ETEC bacteria that express the most important CFs in immunogenic form on the bacterial surface and combine these cells with an appropriate toxoid for inducing LT-neutralizing antibodies, i.e., recombinantly produced CTB or LTB (Holmgren and Svennerholm, 1998; Ahren *et al*, 1993; Svennerholm and Savarino, 2004). Attempts to prepare a strongly immunogenic ST toxoid, either by preparing synthetic peptides coupled to a suitable carrier protein, e.g., recombinant CTB, or by producing recombinant ST-CTB fusion proteins, have not been successful, partly due to the small size and high content of cysteins of the ST molecule. Low-level antibody responses to ST have been possible to raise, but have been found to be insufficient for inducing protection in the gut.

An ETEC vaccine consisting of a combination of recombinantly produced CTB and formalin-inactivated ETEC bacteria expressing five of the most prevalent CFs (CFA/I, CS1-CS5) and common O-antigens (e.g., O6, O25, O78, O139) of ETEC has been produced and extensively studied in clinical trials (Svennerholm and Savarino, 2004). This rCTB-CF ETEC vaccine has been shown to be safe and give rise to significant IgA immune responses in intestinal fluid in 70–90% of vaccinated Swedish volunteers (Ahren *et al*., 1998). Phase I and II trials of the vaccine in Swedish, Bangladeshi, and Egyptian adult volunteers have all shown that it is well tolerated and gives rise to mucosal immune responses, i.e., peripheral blood antibody secreting cell responses against LT and the different vaccine CFs, in 70–100% of the volunteers (Svennerholm and Savarino, 2004; Åhrén *et al*., 1998; Savarino *et al*., 1998, Qadri *et al*., 2000). Analogous safety/ immunogenicity clinical trials with the CTB-WC ETEC vaccine in Egyptian and Bangladeshi children down to six months of age showed that the vaccine was equally immunogenic as in the adults and also well tolerated except in the youngest Bangladeshi infants. A dose finding study showed that a quarter of a full dose of rCTB-CF ETEC vaccine was equally safe and immunogenic in the youngest infants as a full dose in older children and adults (Qadri *et al*., to be published).

The protective efficacy of the rCTB-CF ETEC has been studied in adult travelers going from industrialized areas to different countries in Asia, Africa, and Latin America. In an initial pilot study in European travelers going to 20 different countries in Africa, Asia, and Latin America, the vaccine was shown to confer 82% protective efficacy ($p < 0.05$) against ETEC disease, but the number of cases fulfilling the inclusion criteria overall was low (Wiedermann *et al*., 2000). Clinical trials of the vaccine in European travelers going to Kenya or in Israeli soldiers have not allowed determination or the protective efficacy of the vaccine due to too few cases fulfilling the inclusion criteria (Svennerholm and Savarino, 2004). In a large placebo-controlled trial that was recently completed in nearly 700 American travelers going to Mexico and Guatemala, the rCTB-CF ETEC vaccine was effective (protective efficacy 77%, $p = 0.039$) against non-mild ETEC diarrheal illness, i.e., disease that interfered with the travelers' daily activity, whereas no significant protection was observed against ETEC diarrhea of any severity, including mainly mild cases (Sack *et al*., 2002).

The only pediatric study to assess efficacy of the rCTB-CF ETEC vaccine is a randomized, double-blind, control trial that was recently conducted in 350 6–18-month-old children in rural Egypt (Svennerholm and Savarino, 2004). Disease detection was based on active

surveillance through semi-weekly household visits and cultures of fecal specimens from children with diarrhea. No significant protection against ETEC disease was induced by the vaccine in this trial, but it should be noted that children with high immune responses were protected (Savarino *et al*., to be published). Based on these results from the testing of the vaccine in children in a developing country, there appears to be a need for improving vaccine efficacy.

Since not only LTB but also the immunologically closely related and strongly cross-reactive CTB are efficiently immunogenic, lack toxicity, are stable in the gastrointestinal milieu, and are capable of binding to the intestinal epithelium including so-called M cells on the Peyer's patch surface, both of these proteins are suitable to give rise to protective anti-LT immunity. Although CTB has afforded significant protection against *E. coli* LT disease (Clemens *et al*., 1988b; Peltola *et al*., 1991), an LT toxoid may be slightly more effective than CTB in inducing protective anti-LT immunity (Svennerholm and Savarino, 2004).

An intriguing finding, if it can be confirmed in humans, is that in mice transcutaneous immunization may induce a mucosal immune response.

Based on this, the possibility of immunizing transcutaneously with an *E. coli* colonization factor, CS6, alone or together with nonmutated LT, was evaluated in human volunteers (Guerena-Burgueno *et al*., 2002). No response to CS6 was observed in the absence of LT (which similar to CT is a strong adjuvant), whereas combined administration of the two antigens induced antibody secreting cell responses as well as serum antibody responses against CS6 in about half of the volunteers and anti-LT responses in serum in all of the vaccines (Guerena-Burgueno *et al*., 2002). The approach of giving recombinantly produced *E. coli* LT transcutaneously, either alone or following oral priming with LT antigen, will be further explored in volunteers in developed and in developing countries (Calderwood *et al*., personal communication).

Different strategies to construct live ETEC vaccines have also been attempted in which either attenuated *Shigella* or non-pathogenic *E. coli* are engineered to express different CS components alone or in combination with an LT toxoid. Considerable progress has been achieved recently in engineering strains of *Shigella* that are attenuated compared with their wild-type parent and in using these strains as live vectors to express ETEC CF and toxin antigens. Enlarging upon this strategy, different live multivalent *Shigella*/ETEC hybrid vaccines have been constructed, wherein the important fimbrial CFs are expressed along with mutated LT in attenuated *Shigella* (Altboum *et al*., 2001). Recently an attenuated *Shigella flexneri 2a* strain, CVD 1204, was used to individually express four different CFs, as well as a detoxified version of human LT (LThK63). Following mucosal immunization of guinea pigs with a mixture of two or more such *Shigella* strains, immune responses were observed against LT, the CF antigens, and O-antigen of the *Shigella* vector strain (Barry *et al*., 2003). Different Shigella-ETEC hybrid candidate vaccine strains are presently tested for safety and immunogenicity, both in guinea pigs and in monkeys. The ultimate goal is to produce five different *Shigella* strains that can express the most important CFs and an LT toxoid simultaneously in the gut.

While such developments of live ETEC vaccines may yield promising experimental vaccines, their usefulness in humans has yet to be established. It remains to be shown to what extent the safety and efficacy profiles established in animal models hold true in genetically diverse human subjects. Thus, humans in different geographic areas may differ significantly in their intestinal flora, nutritional status, and previous immunological experience, all of which are factors that have been found to affect mucosal vaccine efficacy. Indeed, several mucosal vaccines, including oral live cholera vaccine and rotavirus vaccine candidates, as well as oral polio vaccine, have been found to work less well in developing country settings than in industrialized countries. Furthermore, the pandemic HIV infection problem presents additional challenges with regard to both vaccine safety and efficacy, especially for live-attenuated vaccines.

## PERTUSSIS TOXIN AND DEVELOPMENT OF ACELLULAR PERTUSSIS VACCINE

Pertussis toxin (PT) is a major virulence factor of *Bordetella pertussis* and plays an important role in the pathogenesis of whooping cough. There are striking resemblances between the structure and function of PT and those described previously for CT and LT. Thus, similar to the latter toxins, PT has an AB5 subunit structure, wherein the A region (usually referred to as the S1 subunit) mediates ADP ribosylating enzymatic-toxic activity, and the B pentamer, the binding to target cells (Stein *et al*., 1994). However, different from the B homopentamers of CT and LT, the non-toxic B domain of PT is a heteropentamer consisting of one S2, one S3, two S4, and one S5 non-covalently linked subunits (Lobet *et al*., 1993). Binding to cell membrane receptors takes place mainly through the S2 and the S3 subunits, leading to translocation of the S1 subunit into the target cell where it can interact with the $G_i$ component of

adenylate cyclase and other GTP-binding proteins and block their activities.

In the last 20 years, there has been a progressive replacement of the efficacious but reactogenic whole-cell pertussis vaccines with different types of acellular vaccines. The latter vaccines usually contain several components, including detoxified forms of PT together with bacterial cell components, such as filamentous hemagglutinin (FHA) and one or more agglutinogens, such as pertactin and fimbrial antigens. Even vaccine containing exclusively PT toxoid has been used and found to be protective, although to a lesser extent than the more complex acellular vaccines (Edwards *et al*., 1995).

The general approach for detoxifying the bacterial antigens, including PT, present in these vaccine formulations has consisted of different chemical treatments, such as using formaldehyde, glutaraldehyde, or hydrogen peroxide (Edwards *et al*., 1995). Since all of these methods carry the risk of a small but significant rate of reversion to toxic activity of PT, it was attractive to develop a genetically engineered, fully stable, and non-toxic PT "toxoid," wherein the toxic-enzymatic activity of the S1 subunit had been eliminated by specific mutation. Among several such mutated PT molecules showing variable degrees of detoxification, one molecule having substitutions in two positions of the S1 subunit (at residues 9 and 129) was found to be totally devoid of toxic-enzymatic activity and thus selected for further testing towards the development of an acellular pertussis vaccine (Pizza *et al*., 1989; Nencioni *et al*., 1990).

Studies in animals confirmed that this mutant PT molecule appeared to be devoid of any of the classical toxic-active manifestations of wild-type PT, and also much better than previous chemically detoxified PT molecules that had preserved epitopes reacting with and inducing neutralizing antibodies. The safety and immunogenicity of the genetically inactivated PT has been extensively tested in clinical trials, both in adult volunteers and in infants and children (Podda *et al*., 1990, 1992, 1993). These trials, both using the mutant PT alone and in association with FHA and pertactin, showed that acellular pertussis vaccines based on engineered, non-toxic PT mutant were safe and induced high titers of anti-PT-neutralizing antibodies, as well as strong PT specific T cell proliferative responses. Further, in a double-blind, placebo-controlled phase III efficacy trial in 16,000 infants in Italy, a three-component acellular pertussis vaccine containing the genetically detoxified PT, FHA, and pertactin given together with diphtheria and tetanus toxoids showed a high level of efficacy (84%) against whooping cough with indirect evidence that the PT component contributed significantly to the efficacy (Pertussis Working Group, 1996).

## DIPHTHERIA TOXIN CRM 197 AS CARRIER IN CONJUGATE VACCINES

Diphtheria toxin (DT) is together with CT the best studied enterotoxin with regard to its structure and function. DT is a 58-kDa protein produced as a single polypeptide by lysogenic *Corynebacterium diphtheriae* strains. After reduction of disulfide bonds, DT consists of two fragments, A and B, responsible for enzymic-toxic and cell-binding properties, respectively (Van Ness *et al*., 1980; Bennett *et al*., 1994). Like the previous toxins discussed, DT ADP-ribosylates an intracellular target protein, for DT the elongation factor 2, which thereby becomes inactive, resulting in inhibition of cellular protein synthesis and cell death.

Already in 1971, Uchida and coworkers (1971) found that different non-toxic DT protein derivatives could be obtained following mutagenesis with nitrosoguanidin. These proteins were called cross-reacting material (CRM) since they were immunologically cross-reactive with DT. Among these mutant proteins, CRM 197, which is mutated in residue 52 in the A fragment, is being widely used as a carrier molecule for the production of conjugated vaccines containing capsular polysaccharides from bacteria (Decker *et al*., 1992; Del Giudice and Rappuoli, 1999). Thus, CRM197 is the carrier molecule for the protective polysaccharide antigen in a licensed *Hemophilus influenzae* conjugate vaccine (Vadheim *et al*., 1994), and is likely to be used also in future conjugate vaccines against pneumococcal and meningococcal infections.

## CT AND LT AND DERIVATIVES AS MUCOSAL ADJUVANTS

Besides being strong mucosal immunogens, both CT and LT are powerful mucosal adjuvants (Holmgren *et al*., 1993). They strongly potentiate the immunogenicity of most other antigens, whether these are linked to or simply admixed with the toxins, provided that the other antigen is given at the same time and at the same mucosal surface as the toxins. However, since both CT and LT are normally considered to be much too toxic for human use, great efforts have recently been made to define whether it would be possible to separate the adjuvant and toxic activities as a basis for development of mucosal adjuvants for human use.

### Adjuvant action of CT and LT

CT and LT can affect several steps in the induction of a mucosal immune response. These effects, which alone or in combination might explain their strong adjuvant action after oral immunization, include: (i) increased permeability of the intestinal epithelium leading to enhanced uptake of co-administered antigen; (ii) enhanced antigen presentation by various APCs; (iii) promotion of isotype differentiation in B cells leading to increased IgA formation; and (iv) complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production. In addition, CT and LT have been shown not only to avoid inducing oral tolerance, but also to abrogate otherwise efficient regimens for tolerance induction by oral antigen administration (Holmgren *et al*., 1993; Lycke, 1997).

Among these many effects, those leading to enhanced antigen presentation by various APC are probably of the greatest importance for the adjuvant activity. CT markedly increases antigen presentation by dendritic cells (DC), macrophages, and B cells and has also been found, at least *in vitro*, to make intestinal epithelial cells to become effective APC (Gagliardi *et al*., 2002). Consistent with this activity, CT upregulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules, as well as chemokine receptors, such as CCR7 and CXCR4 on both murine and human DC and other APC (Gagliardi *et al*., 2002; Eriksson *et al*., 2003). Importantly, CT also induces the secretion of IL-1 from DC (Eriksson *et al*. 2003), thus supporting similar observations made earlier for macrophages. IL-1 not only induces the maturation of DC, but is also by itself an efficient mucosal adjuvant when co-administered with protein antigens and might mediate a significant part of CT's adjuvant activity (Staats and Ennis, 1999).

It has been claimed that CT primarily induces Th2 type immune responses characterized by $CD4^+$ T cells producing IL-4, IL-5, IL-6, and IL-10 and by the production of IgA, IgG1, and IgE antibodies. LT, on the other hand, has been reported to induce a mixed Th1- and Th2-type immune response. However, other studies have shown that CT also can induce mixed Th1 and Th2 types of immune responses, in contrast to CTB, which appears to induce a more restricted Th2 type of immune response (Eriksson *et al*., 2003). Thus DC, which had been pre-treated *in vitro* with a protein antigen (ovalbumin, OVA) linked to or admixed with CT and then injected into mice in an antigen-specific manner, induced both Th1 and CTL responses in addition to a Th2 response. In contrast, DC pulsed *in vitro* with OVA linked to CTB only gave rise to a Th2 type of immune response.

## DEVELOPMENT OF NON-TOXIC DERIVATIVES AS MUCOSAL ADJUVANTS

To avoid toxicity, isolated CTB and LTB have been explored for their ability to augment immune responses also against co-administered antigens. However, their capacity as mucosal adjuvants has proved to be much less than that of the holotoxins. Indeed, both CTB and LTB are poor adjuvants when given to animals together with non-coupled antigens by the oral route, although they display a more significant adjuvant activity when administered via the nasal route. Mice vaccinated intranasally with the influenza virus HA vaccine PR8 H1N1 together with LTB had higher levels of anti-viral IgA and IgG, both in sera and in nasal and lung secretions compared to mice given the subunit vaccine alone, and were also protected against an intranasal viral challenge (Haan *et al*. 2001). Adjuvanticity of CTB or LTB is much improved when coupled to antigens, due to the increased uptake of coupled antigen across the mucosal barrier and the more efficient presentation of coupled antigen, as compared with uncoupled antigen not only by DC and macrophages, but also by naïve B cells (Eriksson *et al*., 2003; George-Chandy *et al*., 2001).

Recently, site-directed mutagenesis has permitted the generation of LT and CT mutants that have reduced toxicity but that retain significant adjuvanticity when given to animals by the nasal-mucosal route, or even though they then perform less well, by the oral-mucosal route (Pizza *et al*., 2001). Two such mutants, LTK63 and LTK72, are currently evaluated as adjuvants for intranasally administered influenza vaccine (A. Podda *et al*., to be published).

Another approach that has been used to circumvent the harmful drawbacks of CT or LT adjuvants is to link the enzymatically active A subunit domain of the toxin to a cell-binding moiety other than the natural B subunit, such as the cell-binding domain of *Staphylococcus aureus* protein A (CTA1-DD) (Ågren *et al*., 1999). CTA1-DD, like most other toxin derivates, functions when applied nasally but not when given orally. This limitation has recently been overcome by the incorporation of CTA1-DD fused to a short peptide into immune stimulating complexes (ISCOMS). Oral vaccination with the ISCOM-CTA1-DD complex induced systemic and mucosal responses with both Th1 and Th2 characteristics (Mowat *et al*., 2001).

To achieve detoxification of CT, yet another type of mutant was recently described in which peptides were added to the CTA1 amino end. The added peptides seemed to reduce both enterotoxicity and ADP-ribosylating activity by sterical hindrance with the

CTA1 active site (Sanchez *et al*., 2002). In general, in these detoxified constructs, adjuvanticity decreased with decreasing enterotoxicity/ADP-ribosylation. However, a mutant (eCT6), with 10- to 20-fold lower enterotoxicity, displayed a level of adjuvanticity comparable to that of the wild-type CT. Another mutant with a longer peptide linked to CTA1 (eCT23) and no detectable toxic activity, although being also much less potent in adjuvant activity than either CT or eCT6, was superior to CTB as both mucosal immunogen and as adjuvant for a co-administered antigen.

## CT AND CTB AS ADJUVANTS FOR DENDRITIC CELL VACCINATION

Dendritic cells (DC) are professional antigen (Ag)-presenting cells that act as sentinels throughout the body. Vaccination with autologous monocyte/bone-marrow-derived DC pulsed with an appropriate Ag together with a suitable adjuvant *ex vivo* represents a promising approach to immunotherapeutic vaccination in patients with cancer or severe infections. The fact that the *ex vivo*-pulsed DC are autologous and can be extensively washed free from any unprocessed Ag and adjuvant before being reinfused to the patient makes it promising to also be able to use even such strong (but toxic) adjuvants as unmodified CT for stimulating the DC to become maximally immunogenic without risks of causing toxic side effects *in vivo*.

Conjugation of Ag to either CT or CTB greatly facilitated MHC class II-restricted Ag presentation by DC associated with both an up-regulated secretion of IL-1β by the pulsed DC and increased expression of CD80 and CD86 on the DC surface (George-Chandy *et al*., 2001). DC pulsed with CT- or CTB-conjugated Ag were found to be superior to DC pulsed with free Ag at inducing CD4+ T cell and B cell responses *in vivo*. However, while CTB-Ag conjugates almost exclusively stimulated immune responses with a Th2 type profile, conjugates based on CT were shown to drive the *in vivo* responses toward a mixed Th1 and Th2 phenotype (Eriksson *et al*., 2003). Vaccination with Ag-CT-pulsed DC, but not DC pulsed with Ag-CTB or Ag alone, also induced Ag-specific CD8+ T cells that produced IFN and were cytotoxic for Ag(OVA)-expressing E.G7 tumor cells *in vitro* and were able to infiltrate and eliminate an already established OVA-expressing E.G7 tumor *in vivo* (Eriksson *et al*., 2004; Sun *et al*, 2004). We propose that the use of CT as a combined carrier-delivery system and adjuvant for DC vaccination represents a novel attractive strategy for therapeutic anti-tumor vaccination, at least for tumors that express sufficient amounts of MHC class I carrying tumor-specific peptide antigens.

## CTB::CPG – A NOVEL POTENT IMMUNO-MODULATING ADJUVANT

Bacterial DNA contains unmethylated CpG motifs, which distinguishes bacterial DNA from vertebrate DNA. Vertebrate genomes uniformly contain a lower frequency of CpG motifs, and these are usually methylated (Krieg, 2002). Vertebrate immune systems appear to have evolved TLR9, which distinguishes bacterial DNA from self DNA. Interactions between unmethylated CpG motifs in bacterial DNA or in synthetic oligodeoxynucleotide (ODN) and TLR9 rapidly activate DCs, splenocytes, monocytes, and macrophages through the Toll/IL-1-receptor signaling pathway (Wagner, 2002). This interaction results in production of Th1-polarizing cytokines such as IFN-γ, IL-1β, tumor necrosis factor-α (TNF-α), and IL-12, which up-regulate co-stimulatory molecules on APCs and activate B cells for proliferation and IL-6 secretion. *In vivo*, systemic administration of CpG DNA was demonstrated to promote NK cell activity, to increase the total number of B lymphocytes in the spleen, and to elevate plasma levels or tissue mRNA expression of IFN-γ, IL-12, and TNF-α. Altogether, CpG DNA induces a predominantly Th1-type immune activation (Krieg, 2002), and CpG DNA has been shown to enhance vaccine immune responses (Krieg and Davis, 2001).

Very strikingly, it has recently been shown that the immunostimulatory effect of CpG DNA is significantly enhanced when chemically conjugated to CTB (Harandi and Holmgren, 2004). When compared for their immunostimulatory effect on murine splenocytes, CpG ODN coupled to CTB induced much stronger chemokine responses than achieved with CpG alone, and when tested on human white blood cells, even more pronounced differences were found. Thus, *in vitro* treatment of human peripheral blood mononuclear cells (PBMC) with an optimal human CpG ODN did not give rise to any appreciable levels of CC chemokine responses, whereas CpG ODN coupled to CTB elicited strong CC chemokine responses by human PBMC. The corresponding optimal murine CpG conjugated to CTB when administered intravaginally also elicited stronger CC chemokine responses in the murine female genital tract mucosa than did the unconjugated CpG ODN. The mechanism(s) of the increased immunostimulatory property of CTB::CpG conjugate compared to CpG ODN alone remains to be defined, but could be due both to increased uptake of

CTB-coupled CpG ODN across the mucosal barrier, as well as directly by the target antigen-presenting cells to increased transport into endosomes in the latter cells, and possibly also to CTB/GM1-directed intracellular transport of CpG into ER not accomplished by the free CpG ODN (Adamsson *et al*., to be published).

## REFERENCES


Ågren, L.C., Ekman, L., Lowenadler, B. and Nedrud, J.G. (1999). Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. *J. Immunol*. **162,** 2432–2440.

Åhrén, C., Jertborn, M. and Svennerholm, A-M. (1998). Intestinal immune responses to an inactivated oral enterotoxigenic *Escherichia coli* vaccine and associated immunoglobulin A responses in blood. *Infect. Immun*. **66,** 3311–3316

Altboum, Z., Barry, E.M., Losonsky, G., Galen, J.E. and Levine, M.M. (2001). Attenuated *Shigella flexneri 2a* Delta *guaBA* strain CVD 1204 expressing enterotoxigenic *Escherichia coli* (ETEC) CS2 and CS3 fimbriae as a live mucosal vaccine against *Shigella* and ETEC infection. *Infect. Immun*. **69,** 3150–3158.

Barry, E.M., Altboum, Z., Losonsky, G. and Levine, M.M. (2003). Immune responses elicited against multiple enterotoxigenic *Escherichia coli* fimbriae and mutant LT expressed in attenuate *Shigella* vaccine strains. *Vaccine* **21,** 333–340.

Bennett, M.J., Choe, S. and Eisenberg, D. (1994). Refined structure of dimeric diphtheria toxin at 1.0 Angstrom resolution. *Protein Sci*. **3,** 1444–1463.

Black, R.E., (1990). Epidemiology of travelers' diarrhea and relative importance of various pathogens. *Rev. Infect. Dis*. **1,** 73–79.

Black, R.E. (1993). Epidemiology of diarrheal disease: implications for control by vaccines. *Vaccine* **11,** 100–106.

Clemens J.D., Sack D.A., Harris J.R., Chakraborty J., Khan M.R., Stanton B.F., Kay B.A., Khan M.U., Yunus M., Atkinson, W., Svennerholm, A.M. and Holmgren, J. (1986). Field trial of oral cholera vaccines in Bangladesh. *Lancet*. **19,** 2124–7.

Clemens, J.D. Harris, J.R., Sack, D.A. Chakraborty, J., Ahmed, F., Stanton, B.F., Khan, M.U., Kay, B.A., Huda, N., Khan, M.R., Yunus, M., Rao, M.R., Svennerholm, A.M. and Holmgren, J. (1988a). Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. *J. Infect. Dis*. **158,** 60–69.

Clemens, J.D., Sack, D.A., Harris, J.R., Chakraborty, J., Neogy, P.K., Stanton, B., Huda, N., Khan, M.U., Kay, B.A., Khan, M.R., Kay, B.D., Khan, M.R., Ansaruzzamen, M., Yunus, M., Rao, M.R., Svennerholm, A-M. and Holmgren, J. (1988b). Cross-protection by B subunit-whole cell cholera vaccine against diarrhea associated with heat-labile, toxin-producing enterotoxigenic *Escherichia coli*: results of a large-scale field trial. *J. Infect. Dis*. **158,** 372–377.

Clemens, J.D., Sack, D.A., Harris, J.R., Van Loon, F., Chakraborty, J., Ahmed, F., Rao, M.R., Khan, M.R., Yunus, M., Huda, N., Stanton, B.F., Kay, B.A., Walter, S., Eckels, R., Svennerholm, A.M. and Holmgren, J. (1990). Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. *Lancet* **335,** 270–273.

Cohen, M.B., Giannella, R.A., Bean, J., Taylor, D.N., Parker, S., Hoeper, A., Wowk, S., Hawkins, J., Kochi, S.K., Schiff, G. and Killeen, K.P. (2002). Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. *Infect. Immun*. **70,** 1965–1970.

Czerkinsky, C., Anjuere, F., McGhee, J.R., George-Chandy, A., Holmgren, J., Kieny, M.P., Fujiyashi, K., Mestecky, J.F., Pierrefite-Carle, V., Rask, C. and Sun, J.B. (1999). Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev*. **170,** 197–222.

Decker, M.D., Edwards, K.M., Bradley, R. and Palmer, P. (1992). Comparative trials in infants of four conjugate *Haemophilus influenzae* type B vaccines. *J. Pediatr*. **120,** 184–189.

Edwards, K.E., Meade, B.D., Decker, M.D., Reec, G.F., Rennels, M.B., Steinhoff, M.C., Anderson, E.L., Englund, J.A., Pichichero, M.E., Deloria, M.A. and Deforest, A. (1995). Comparison of 13 acellular pertussis vaccines: overview and serologic response. *Pediatrics* **96,** 548–557.

Eriksson, K., Fredriksson, M., Nordström, I. and Holmgren, J. (2003). Cholera toxin and its B subunit promote dendritic cell vaccination with different influence on Th1/Th2 development. *Infect. Immun*. **71,** 1740–1747.

Eriksson, K., Sun, J.B., Nordström, I., Fredriksson, M., Lindblad, M., Li, B.L. and Holmgren,J. (2004). Coupling of antigen to cholera toxin for dendritic cell vaccination promotes the induction of MHC class I-restricted cytotoxic T cells and the rejection of a cognate antigen-expressing model tumor. *Eur. J. Immunol*. **34,** 1272–1281.

Gaastra, W. and Svennerholm, A.M. (1996). Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol*. **4,** 444–452.

Gagliardi, M., Sallusto, F., Marinaro, M., Vendetti, S., Riccomi, A. and De Magistris, M. (2002). Effects of the adjuvant cholera toxin on dendritic cells: stimulatory and inhibitory signals that result in the amplification of immune responses. *Int. J. Med. Microbiol*. **291,** 571–575.

George-Chandy, A., Eriksson, K., Lebens, M., Nordstrom, I., Schon, E. and Holmgren, J. (2001). Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. *Infect. Immun*. **69,** 5716–5725.

Guerena-Burgueno, F., Hall, E.R., Taylor, D.N., Cassels, F.J., Scott, D.A., Wolf, M.K., Roberts, Z.J., Nesterova, G.V., Alving, C.R. and Glenn, G.M. (2002). Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. *Infect. Immun*. **70,** 1874–1880.

Guerrant, R.L. (1985). Microbial toxins and diarrheal disease: introduction and overview. In: *Microbial Toxins and Diarrheal Disease*. Ciba Foundation Symp. 112. pp. 1–13. Pitman, London.

Del Giudice, G. and Rappuoli, R. (1999). Genetically derived toxoids for use as vaccines and adjuvants. *Vaccine* **17,** 44–52.

De Haan, L. and Hirst, T.R. (2000). Cholera toxin and related enterotoxins: a cell biological and immunological perspective. *J. Nat. Toxins* **9,** 281–297.

Harandi, A.L. and Holmgren, J. (2004). CpG DNA as a potent inducer of mucosal immunity: implications for immunoprophylaxis and immunotherapy of mucosal infections. *Curr. Opin. Investig. Drugs* **5,** 141–5.

Holmgren, J. (1981). Actions of cholera toxin and the prevention and treatment of cholera. *Nature* **292,** 413–417.

Holmgren, J. and Bergquist, C. (2004). Oral B subunit killed whole-cell cholera vaccines. In: *New Generation Vaccines, 3rd* ed. (eds. Levine M.M. *et al*.) pp. 499–510. Marcel Decker, New York.

Holmgren, J. and Czerkinsky, C. (2005). Mucosal immunity and vaccines. *Nature Med*., in press.

Holmgren, J., Lycke, N. and Czerkinsky, C. (1993). Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector system. *Vaccine* **11,** 1179–1184.

Holmgren, J. and Svennerholm, A.M. (1998). Vaccines against diarrheal disease. In: *Handbook of Experimental Pharmacology* (eds. P. Perlmann and H. Wigzell), pp. 291–328, Berlin-Heidelberg, New York.

Holmgren, J., and Svennerholm, A-M. (2004). Mucosal immunity and bacteria. In: *Mucosal Immunology* 3rd edition, eds. Mestecky, J. *et al*, London, Academic Press, Elsevier Science, in press.

Holmgren, J., Svennerholm, A.M., Lönnroth, I., Fall-Person, M., Markman, B. and Lundbäck, H. (1977). Development of improved cholera vaccine based on subunit toxoid. *Nature* **269,** 602–604.

Jertborn, M., Svennerholm, A.M. and Holmgren, J. (1992). Safety and immunogenicity of an oral recombinant cholera B subunit-whole cell vaccine in Swedish volunteers. *Vaccine* **10,** 130–132.

Kenner, J.R., Coster, T.S., Taylor, D.N., Trofa, A.F., Barrera-Oro, M., Hyman, T., Adams, J.M., Beattie, D.T., Killeen, K.P. and Spriggs, D.R. (1995). Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. *J. Infect. Dis*. **172,** 1126–1129.

Krieg, A.M. and Davis, H. (2001). Enhancing vaccine with immune stimulatory CpG DNA. *Curr. Opin. Mol. Ther*. **3,** 15–24.

Krieg, A.M. (2002). CpG motifs in bacterial DNA and their immune effects. *Ann. Rev. Immunol*. **20,** 709–760.

Levine, M.M. and Kaper, J.B. (1995). Live oral cholera vaccine: from principle to product. *Bull. Inst. Pasteur* **93,** 243–253.

Levine, M.M. and Tacket, C.O. (1995). Live oral vaccines against cholera. In: *Molecular and Clinical Aspects of Bacterial Vaccine Development* (eds. D.A.A. Ala'Aldeen and C.E. Hormaeche), pp. 233–258. John Wiley and Sons, UK.

Lebens, M., Johansson, S., Osek, J., Lindblad, M. and Holmgren, J. (1993). Large-scale production of Vibrio cholerae toxin B subunit for use in oral vaccines. *Biotechnology*, **11,** 1574–1578.

Lieberman, J.M., Chiu, S.S., Wong, V.K., Partridge, S., Chang, S.J., Chiu, C.Y., Gheesling, L.L., Carlone, G.M. and Ward., J.I. (1996). Safety and immunogenicity of a serogroup A/C *Neisseria meningitides* oligosaccharide-protein conjugate vaccine in young children: a randomized controlled trial. *JAMA* **275,** 1499–1503.

Lobet, Y., Feron, C., Desquesne, G., Simoen, E., Hauser, P. and Locht, C. (1993). Site-specific alterations in the B oligomer that affect receptor-binding activities and mitogenicity of pertussis toxin. *J. Exp. Med*. **177,** 79–87.

Lucas, M., Deen J.L. and von Seidlein, L. *et al*. (2005). High-level effectiveness of a mass cholera vaccination in Beira, Mozambique. *N. Eng. J. Med*. (in press).

Lycke, N. (1997). The mechanism of cholera toxin adjuvanticity. *Res. Immunol*. **148,** 405–520.

Medzhitov, R. and Janeway, C. Jr. (2000). The toll receptor family and microbial recognition. *Trends. Microbiol*. **8,** 452–456.

Mowat, A.M., Donachie, A.M., Jagewall, S., Schon, K., Lowenadler, B., Dalsgaard, K., Kaastrup, P. and Lycke, N. (2001). CTA1-DD-immune stimulating complexes: a novel, rationally designed combined mucosal vaccine adjuvant effective with nanogram doses of antigen. *J. Immunol*. **167,** 3398–3405.

Nencioni, N., Pizza., M., Bugnoli, M., de Magistris, T., Di Timmaso, A., Giovannoni, F., Manetti, R., Marsili, I., Matteucci, G., Nucci, D., Olivieri, R., Pileri, P., Presentini, R., Villa, L., Kreftenberg, J.G., Silvestri, S., Tagliabue, A. and Rappuoli, R. (1990). Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. *Infect. Immun*. **58,** 1308–1315.

Neutra, M.R. and Kraehenbuhl, J.P. (1993). The role of transepithelial transport by M cells in microbial invasion and host defense. *J. Cell Sci*. **17,** S209–215.

Peltola, H., Siitonen, A., Kyronseppa, H., Simula, I., Mattila, L., Oksanen, P, Kataja, M.J. and Cadoz, M. (1991). Prevention of travelers' diarrhea by oral B-subunit/whole-cell cholera vaccine. *Lancet* **338,** 1285–1289.

Pizza, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L, de Magistris, M.T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., Giovannoni, F., Olivieri, R., Barbieri, J.T., Sato, H. and Rappuoli, R. (1989). Mutants of pertussis toxin suitable for vaccine development. *Science* **246,** 497–500.

Pizza, M., Giuliani, M., Fontana, M., Monaci, E., Douce, G., Dougan, G., Mills, K.H., Rappuoli, R. and Del Giudice, G. (2001). Mucosal vaccines: non-toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* **19,** 2534–2541.

Podda, A., Carapella de Luca, E., Titone, L., Casadei, A.M., Cascio, A., Peppoloni, S., Volpini, G., Marsili, I., Nencioni, L. and Rappuoli, R. (1992). Acellular pertussis vaccine composed of genetically inactivated pertussis toxin: safety and immunogenicity in 12- to 24- and 2- to 4-month-old children. *J. Pediatr*. **120,** 680–685.

Podda, A., Carapella de Luca, E., Titone, L., Casadei, A.M., Cascio, A., Bartalini, M., Volpini, G., Peppoloni, S., Marsili, I., Nencioni, L. and Rappuoli, R. (1993). Immunogenicity of an acellular pertussis vaccine composed of genetically inactivated pertussis toxin combined with filamentous hemagglutinin and pertactin in infants and children. *J. Pediatr*. **123,** 81–84.

Podda, A., Nencioni, L., de Magistris, M.T., Di Tommaso, A., Bossu, P., Nuti, S., Pileri, P., Peppoloni, S., Bugnoli, M., Ruggiero, P., Marsili, I., D'Errico, A., Tagliabue, A. and Rappuoli, R. (1990). Metabolic, humoral, and cellular responses in adult volunteers immunized with the genetically inactivated pertussis toxin mutant PT-9K/129G. *J. Exp. Med*. **172,** 861–868.

Pertussis Working Group, Greco, D., Salmaso, S., Mastrantonio, P., Giuliano, M., Tozzi, A.F., Anemona, A., Ciofi degli Atti, M.L., Giammanco, A., Panei, P., Blackwelder, W.C., Klein, D.L. and Wassilak, S.G.F. (1996). A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. *N. Engl. J. Med*. **334,** 341–346.

Qadri, F., Wenneras, C., Ahmed, F., Asaduzzaman, M., Saha, D., Albert, M.J., Sack, R.B. and Svennerholm, A.M. (2000). Safety and immunogenicity of an oral, inactivated, enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children. *Vaccine* **18,** 2704–2712.

Richie, E., Punjabi, N.H., Sidharta, Y.Y., Peetosutan, K.K., Sukandar, M.M., Wasserman, S.S., Lesmana, M.M., Wangsasaputra, F.F., Pandam, S.S., Levine, M.M., O'Hanley, P.P., Cryz, S.J. and Simanjuntak, C.H. (2000). Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. *Vaccine* **18,** 2399–2410.

Sack, D.A., Shimko, J., Torres, O., Gomis, G., Gustafsson, B., Karnell, A., Nyqvist, I. and Svennerholm, A-M. (2002). Safety and efficacy of a killed oral vaccine for enterotoxigenic *E. coli* diarrhea in adult travelers to Guatemala and Mexico. 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy. San Diego, CA.

Sack, R.B. (1980). Enterotoxigenic *Escherichia coli*: identification and characterization. *J. Infect. Dis*. **142,** 279–286.

Sanchez, J. and Holmgren, J. (1989). Recombinant system for overexpression of cholera toxin B subunit in Vibrio cholerae as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA* **86,** 481–485.

Sanchez, J., Wallerstrom, G., Fredriksson, M., Angstrom, J. and Holmgren, J. (2002). Detoxification of cholera toxin without removal of its immunoadjuvanticity by the addition of (STa-related) peptides to the catalytic subunit. A potential new strategy to generate immunostimulants for vaccination. *J. Biol. Chem*. **277,** 33369–33377.

Sanchez, J.L., Vasques, B., Begue, R.E., Meza, R., Castellares, G., Cabezas, C., Watts, D.M., Svennerholm, A-M., Sadoff, J.C. and Taylor, D.N. (1994). Protective efficacy of oral whole-cell/recombinant-B-subunit cholera vaccine in Peruvian military recruits. *Lancet* **344,** 1273–1276.

Savarino, S.J., Brown, F.M., Hall, E., Bassily, S., Youssef, F., Wierzba, T., Peruski, L., El-Masry, N.A., Safwat, M., Rao, M., Jertborn, M., Svennerholm, A.M., Lee, Y.J. and Clemens, J.D. (1998). Safety and immunogenicity of an oral, killed enterotoxigenic *Escherichia*

*coli*—cholera toxin B subunit vaccine in Egyptian adults. *J. Infect. Dis*. **177,** 796–799.

Scerpella, E.G., Sanchez J.L., Mathewson J.J., III, Torres-Cordero J.V., Sadoff J.C., Svennerholm A.M., DuPont H.L., Taylor D.N., and Ericsson C.D. (1995). Safety, immunogenicity, and protective efficacy of the whole-cell/recombinant B subunit (WC/rBS) oral cholera vaccine against travelers' diarrhea. *J. Travel Med*. **2,** 22–27.

Staats, H.F. and Ennis, F.A. Jr. (1999). IL-1 is an effective adjuvant for mucosal and systemic immune responses when co-administered with protein immunogens. *J. Immunol*. **162,** 6141–6147.

Stein, P.E., Boodhoo, A., Armstrong, G.D., Cockle, S.A., Klein, M.H. and Read, R.J. (1994). The crystal structure of pertussis toxin. *Structure* **2,** 45–57.

Sun, J., Eriksson, K., Linblad, M., Azem, J. and Holmgren, J. (2004). Vaccination with dendritic cells pulsed *in vitro* with tumor antigen conjugated to cholera toxin efficiently induces specific tumoricidal CD8+ cytotoxic lymphocytes dependent on cyclic AMP activation of dendritic cells. *Clin. Immunol*. 11235–44.

Svennerholm, A.M. and Holmgren, J. (1976). Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. *Infect. Immun*. **13,** 735–740.

Svennerholm, A.M. and Savarino, S.J. (2004) Oral inactivated whole cell B subunit combination vaccine against enterotoxigenic *Escherichia coli*. In: *New Generation Vaccines* 3rd ed., (eds. M.M. Levine *et al*.,) pp. 737–750. Marcel Decker, New York.

Svennerholm, A.M. and Steele, D. (2004). Host-enteropathogen interactions in the gut: Progress in enteric vaccine development. In: *Microbial-Gut Interactions in Health and Disease* (ed. M. Farthing.) *Clin. Gastroenterol*. **18,** 421–445.

Tayot, J.L., Holmgren, J., Svennerholm, L., Lindblad, M. and Tardy, M. (1981). Receptor-specific, large-scale purification of cholera toxin on silica beads derivatized with lysoGM1 ganglioside. *Eur. J. Biochem*. **113,** 249–258.

Uschida, T., Gill, D.M. and Pappenheimer, A.M., Jr. (1971). Mutation in the structural gene for diphtheria toxin carried by temperate phage. *Nature* **233,** 8–11.

Vadheim, C., Greenberg, D., Eriksen, E., Hemenway, L., Christenson, P., Ward, B., Mascola, L. and Ward, J.I. (1994). Protection provided by *Haemophilus influenzae* type B conjugate vaccines in Los Angeles: a case-control study. *Pediatr. Infect. Dis. J*. **13,** 274–80.

Wagner, H. (2002). Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. *Curr. Opin. Immunol*. **5,** 62–69.

Van Ness, B.G., Howard, J.B. and Bodley, J.W. (1980). ADP-ribosylation of elongation factor 2 by diphtheria toxin. *J. Biol. Chem*. **255,** 10710–10716.

Wiedermann, G., Kollaritsch, H., Kundi, M., Svennerholm, A.M. and Bjare, U. (2000). Double-blind, randomized, placebo-controlled pilot study evaluating efficacy and reactogenicity of an oral ETEC B-subunit-inactivated whole cell vaccine against travelers' diarrhea (preliminary report). *J. Travel Med*. **7,** 27–29.

World Health Organization. Cholera vaccines. (2001). WHO position paper. *Weekly Epidemiol. Res*. **76,** 117–124.

Wu, H.Y. and Weiner, H.L. (2003). Oral tolerance. *Immunol. Res*. **28,** 265–284.

CHAPTER

# 62

# Bacterial protein toxins as biological weapons

*Leonard A. Smith*

## INTRODUCTION: EVOLUTION OF BIOLOGICAL WEAPONRY

Naturally occurring diseases have plagued civilization longer than recorded history, as ancient civilization existed before primordial observations and accounts were transcribed and maintained. The impact of disease on civilization has influenced the course of history and continues to do so in many different ways. Since antiquity, the war fighters and designers of battlefield tactics seized upon nature's disease-causing microbes and viruses and used them as weapons to help defeat their enemies in war. Accounts of biowarfare date back centuries B.C. when the early Persians, Greeks, and Romans contaminated the water supplies of their enemies with poisons from plants, animal carcasses, cadavers, and any refuse available that could cause disease in their opponents (Warner, 1972; Kupperman and Smith, 1993; Barnaby, 1997; Eitzen and Takafuji, 1997; Christopher *et al*., 1997). Further examples of biowarfare are found throughout history and have continued into the 21st century. In 1346, the invading Tartar army catapulted plague-infected corpses into the Genoese-held city of Kaffa (present day Feodosia in the Ukraine) during their attack on the Crimean city in an attempt to cause an epidemic within enemy forces (Derbes, 1966; Geissler, 1986; Mee, 1990). This maneuver (i.e., hurling bodies of people who had died of bubonic plague) was repeated in 1710 when Russians attacked Swedish forces at Reval in Estonia (Barnaby, 1997). Smallpox was used as a biological agent during the French and Indian War (1754–1767) (Stearn and Stearn, 1945; Parkman, 1969; Cartwright, 1974; Geissler, 1986; Cole, 1988; Poupard *et al*., 1989; Wheelis, 1999), the American Revolution (1775–1783) (Eitzen and Takafuji, 1997; Fern, 2000), and World War II (1941–1945) (Harris and Paxman, 1982; Williams and Wallace, 1989). One example of smallpox's use as a biological weapon occurred in 1763 when the British military provided blankets and handkerchiefs contaminated with smallpox from British victims at Fort Pitt to Native Americans with the intent to spread the disease among them. During the French and Indian War, Fort Pitt (today Pittsburg), an outpost in western colonial Pennsylvania, was in danger of being seized by the local Indians, who had already attacked the nearby outpost and killed the inhabitants. The use of smallpox was a covert attempt to infect the Indian population and reduce their numbers.

During World War I, the Germans allegedly developed and used *Bacillus anthracis*, *Pseudomonas pseudomallei*, *Burkholderia pseudomallei*, and *Vibrio cholerae* to cause anthrax, glanders, and cholera. However, Germany denied these assertions and allegations were never proven (Eitzen and Takafuji, 1997). The use of biological agents as weapons against humanity took on new meaning before and during World War II when the Imperial Japanese Army under General Shiro Ishii initiated a massive program to develop, test, and employ biological warfare weapons (Williams and Wallace, 1989; Harris, 1994; Gold, 1996). In 1932, during the Japanese occupation of Manchuria, Ishii began the atrocious program of testing various

disease agents on human subjects in the provincial capital of Harbin, northeast China. In 1936, the secret biowarfare research Unit 731, operating under the guise of a water purification unit, was created 20 miles from Harbin, at Ping Fan. The capacious base situated on approximately six square kilometers included 150 buildings with five satellite camps, and some 300 Japanese scientists and 2,700 technical support staff (Harris, 2003). Their mission was to investigate, among other things, the effects of various pathogens on humans. Three of Unit 731's eight divisions were singly concerned with bacteriological research, warfare research, and the mass production and storage of bacteria. The Japanese military experimented with biological agents, including those that cause anthrax, botulism, brucellosis, dysentery, typhus, gas gangrene, cholera, meningococcal infections, bubonic plague, and smallpox, and their vectors (especially insects), along with potential antidotes and the effects of other drugs, chemical toxins, frostbite, and plant and animal diseases. The experiments used captives, disdainfully referred to as *marutas* or logs, to test the lethal potency of various pathogens in open areas. Victims were bound to stakes on a test site and exposed to cultures in experiments to evaluate the efficacy of biological weapons and delivery methods. In some cases, a disease pathogenesis would be assessed by performing surgery on prisoners exposed to an agent without anesthesia.

In Ping Fan (Unit 731) alone, it is believed that at least 3,000 prisoners of war were killed by biowarfare experimentation during 1941–1945 (Harris, 1997). Thousands more were murdered in the biowarfare death factories in Canton, Peking, Shanghai, Singapore, Mukden, Nanking, Changchum, and in a number of branch camps throughout Manchuria. Even after the war ended, plague epidemics erupted in and around Ping Fan and Harbin proper. In 1947, a major plague epidemic broke out in the same area, spreading through much of the northeast. More than 30,000 persons died from plague before the disease ran its course. A separate facility south of Chang-Chun in Manchuria, called the Army Military Horse Epidemic Prevention Department (Unit 100), adapted agents into delivery devices such as bombs, artillery shells, and spray tanks from aircraft.

From 1940 to 1943, the United Kingdom, the former Soviet Union, and the United States began an extensive research and development program into biological agents. Early in their programs, all three countries ascertained that anthrax could be a viable weapon against the Third Reich after open-field testing in which domestic animals died of pulmonary anthrax after being exposed to anthrax spores by aerosol delivery. These experiments were performed by the British on the Scottish island of Gruinard between 1942–43 and on Vozrozhdeniye Island in the Aral Sea by the Soviet Union around the same period. The Allies' position regarding the use of biological weapons was as a deterrent only to be used in retaliation for an enemy's employment of such a weapon, a policy of limitation said to be in line with the 1925 Geneva Protocol. Ironically, although the Japanese Imperial Army had developed biological weapons and used them against the Chinese and the Russians before and during World War II, the Germans had not devoted significant resources to developing biological weapons, and the U.S. and U.K. never resorted to using them during World War II.

After World War II, the biological weapons programs (both offensive and defensive) of the U.S., the U.K., and the Soviet Union continued to expand at an alarming rate. Finally, in July of 1969, responding to international political pressure, the U.K. proposed a prohibition on the development, production, and stockpiling of bacteriological and toxin weapons at the Conference of the United Nations Committee on Disarmament. Soon after, the Soviet Union proposed that the U.N. General Assembly convene a disarmament convention. In 1972, the U.N. Convention on the Prohibition of the Development, Production, and Stockpiling of Biological and Toxin Weapons and on Their Destruction, which banned the development, production, and stockpiling of biological weapons, was opened for signature (Wright, 1990). The convention entered into force with 43 parties on March 26, 1975, upon ratification by the three depositary states: the U.S., the U.K., and the Soviet Union. As of 2003, the Biological Weapons Convention (BWC) had 151 members who signed and ratified the articles of the BWC treaty and 16 other countries that have signed, but not yet ratified, the treaty. Before the 1972 BWC, the U.S. had stopped all offensive biological and toxin weapon research, development, and production as directed in President Richard M. Nixon's 1969 and 1970 executive orders to do so. Subsequently, all existing stockpiles of biowarfare agents in the U.S. were destroyed in 1971–72. Since 1972 however, the world has witnessed the continued development, stockpiling, and use of biological weapons from nation states and members of the BWC (e.g., the Soviet Union, Iraq) (Meselson *et al*., 1994; Zilinskas, 1997; Butler, 2000; Alibek, 2000), cults (e.g., Rajneeshees, Aum Shinrikyo) (Torok, *et al*., 1997; Carus, 2000, 2001; Sugishima, 2003), and individuals (e.g., Diane Thompson, Debra Green) alike (Carus, 2001; Kolavic, *et al*., 1997).

## POST 9/11 ERA: THE U.S. RESPONSE TO BIOLOGICAL TERRORISM

Events on September 11, 2001 ushered in an acute awareness in the U.S. that its people and homeland were vulnerable to catastrophic terrorist attacks. The recognition was further validated during the same month in 2001 when *Bacillus anthracis* spores were placed in at least four, and potentially seven or more, envelopes and mailed from Trenton, New Jersey, to New York, Washington, D.C., and Lantana, Florida. Two of these letters were addressed to Senators Daschle and Leahy (U.S. Senate) and two others were sent to icons within the media industry. The now infamous anthrax-containing, posted letters, resulted in 22 confirmed or presumed cases of anthrax infection. Of these, 11 cases were inhalation anthrax, and 11 cases of cutaneous anthrax (seven confirmed and four suspected). There were five fatalities among the victims diagnosed with inhalation anthrax. The Centers for Disease Control and Prevention (CDC) recommended that some 10,000 people potentially exposed to anthrax take antibiotics for 60 days. However, one study reported a dramatic increase in prescriptions in October 2001 for ciprofloxacin (up by 160,000 prescriptions or 40%) and doxycycline (up by 120,000 or 30%) over the previous year (Shaffer *et al*., 2003). The increased purchases of post-exposure prophylaxis appeared to reflect the mass panic among the medical community and the public. The cost for anthrax cleanup in Capital Hill offices was $27 million and over $100 million to decontaminate U.S. post offices in Brentwood and New Jersey. To date, there have been no arrests in connection with the anthrax letters, highlighting the difficulty in solving a biocrime of this kind even with today's state-of-the-art forensic capabilities.

## BACTERIAL PROTEIN TOXINS AS BIOLOGICAL WEAPONS

Biological weapons hold many attractions for terrorists, be they renegade groups or nation states, and it is extraordinarily difficult to control their development, manufacture, and delivery. However, there are obvious challenges to the production and successful use of biological weapons whether they are viruses, bacteria, rickettsia, or biological toxins. This chapter focuses on bacterial protein toxins and examines their potential as biological weapons. Biological toxins are molecules produced by living organisms that are poisonous to other species, such as humans. Toxins are not especially effective biological warfare weapons when delivered by aerosols in open-field battlefield situations. They are not infective and do not replicate in the human body as viruses and bacteria do. However, there are scenarios in which certain toxins could have a catastrophic impact on the public health if used in a bioterrorist attack. There are specific features of toxin weapons that make some better weapons than others. In general, an ideal toxin weapon will (i) have a vulnerable population that is unprotected or for which there is no or limited medical intervention available once the disease cause by the toxin has developed; (ii) possess a high specific toxicity or be virulent in low doses; (iii) have a short and predictable incubation period; (iv) be readily mass-produced; (v) remain potent during storage, transport, and dissemination; and (vi) be effectively delivered to its target(s). Although there are numerous bacterial protein toxins capable of causing disease, in many cases the pathogenic organisms or their spores are the most effective delivery vehicle for the toxins and therefore, the organisms or their spores constitute the biological weapon. For example, in the case of inhalational anthrax, *Bacillus anthracis* spores are typically the infective form in the bacterial life cycle. The infective dose by aerosol is 8,000 to 50,000 spores and the incubation period (i.e., time from exposure to symptomatology) is 1 to 5 days (Franz and Zajtchuk, 2000). The spores are able to colonize the human lower respiratory tract, and pulmonary macrophages transport them to the tracheobronchial or mediastinal lymph nodes where they germinate into vegetative cells. As the organisms grow and propagate, they produce anti-phagocytic capsules and at least three bacterial protein toxins (i.e., protective antigen, lethal factor, and edema factor) that play a major role in the anthrax disease. The protective antigen serves as the requisite chaperone molecule for the edema and lethal factors and enables these two toxins to be internalized into cells. These toxins cause necrosis of lymphatic tissue, resulting in the release of large numbers of *Bacillus anthracis* organisms into the circulation, causing a fatal septicemia with widespread hemorrhage and necrosis involving multiple organs.

The CDC through their Bioterrorism Preparedness and Response Office conducted a public health assessment of potential terrorism agents in 1999 (Rotz *et al*., 2002). Their objective was to identify the biological agents towards which preparedness and response efforts in the U.S. were to be initially directed. The assessment of potential terrorism agents was made based on the impact such specific biological agents would have on public health and the medical

infrastructure if they were to be used in a biological attack. Other criteria considered also included the delivery potential to large populations based on the stability of the agent and the ability to produce, transport, and disseminate the agent in quantities adequate to affect such populations, the potential for person to person transmission and/or the degree of infectivity associated with the agent, public perception as related to public fear and potential for civil disruption, and special health preparedness demands for vaccine and therapeutic drug inventories, enhanced surveillance, and diagnostic requirements. The military had previously made similar risk assessments to a multitude of chemical and biological agents based on battlefield vulnerability to its troops.

Based on the assessment study, the CDC categorized biological threat agents in three groups (A, B, and C). Agents in Category A are agents that present the greatest threat to the public health because they are capable of causing mass casualties and are highly contagious or relatively easy to disseminate. These include the etiologic agents causing smallpox (*Variola major*), plague (*Yersinia pestis*), tularemia (*Francisella tularensis*), viral hemorrhagic fever (filoviruses and arenaviruses), anthrax (*Bacillus anthracis*), and botulism (*Clostridium botulinum* neurotoxin). Agents in Category B cause moderate morbidity, result in low mortality rates, and are moderately easy to disseminate. Included in this category are *Coxiella burnetti* (Q-fever); *Brucella* species (brucellosis), *Burkholderia mallei* (glanders), *Burkholderia pseudomallei* (melioidosis); *Chlamydia psittaci* (psittacosis); *Rickettsia prowazekii* (typhus fever); food and water safety threats such as *Salmonella* species, *Shigella* species, *Escherichia coli* O157:H7, and *Vibrio cholerae*; viral encephalitis (e.g., alphaviruses); and three protein toxins: ricin from the plant *Ricinus communis*, enterotoxins from *Staphylococcus aureus*, and epsilon toxin from *Clostridium perfringens*. Category C agents include emerging infectious threats such as Nipah virus and hantavirus.

Three bacterial protein toxins are identified in the CDC select agent list and all three toxins originate from foodborne pathogens. They are botulinum neurotoxin (Category A), staphylococcal enterotoxin (Category B), and epsilon toxin from *C. perfringens* (Category B). Botulinum neurotoxin is the most potent toxin known, its production usually presents few unmanageable difficulties, and it is relatively stable during storage, transport, and various types of dissemination. For these reasons, botulinum neurotoxin has traditionally been the toxin of choice for nation states and terrorists seeking to develop and potentially employ toxins as biological weapons. Much of the remaining chapter will be spent on botulinum neurotoxin because it poses the greatest threat as a toxin weapon and is the only class of toxins classified as Category A threat agents.

## *Clostridium botulinum* neurotoxins

Botulism is a neuroparalytic disease caused by toxins elicited from the saprophytic bacteria *Clostridium botulinum*, *Clostridium baratii*, and *Clostridium butyricum* (CDC, 1998). *C. botulinum* elicits seven structurally similar, but immunologically distinct neurotoxins (about 150 kDa in mass), identified as serotypes A–G (Sugiyama, 1980; Hatheway, 1992). The unique strain *C. baratii* produces only serotype F (Hall *et al*., 1985) and the *C. butyricum* strain, serotype E (Aureli *et al*., 1986). The seven neurotoxins can be differentiated serologically by specific neutralization with antitoxin (Hatheway, 1992). Four of the seven neurotoxins (A, B, E, and F) cause naturally occurring human botulism (Arnon *et al*., 2001), but all seven are known to cause inhalational botulism in primates (Middlebrook and Franz, 1997; J. Anderson, manuscript in preparation), types C and D cause botulism in primates when ingested (Gunnison and Meyer, 1930; Dolman and Murakami, 1961; Smart *et al*., 1980), and type C toxin was shown to be very effective when used in human clinical trials treating patients for blepharospasm and cervical dystonia that had become resistant to treatment with type A neurotoxin (Eleopra *et al*., 2002). The seven neurotoxins have varying specific toxicities (Lamanna, 1959; Gill, 1982; Ohishi, 1984) and durations of persistence in the nerve cells (de Paiva *et al*., 1999; Foran *et al*., 2003), and it is reasonable to assume that all seven toxins can cause botulism in humans if the exposure level is high enough. Portals of entry for botulinum neurotoxin include the pulmonary tract (inhalation botulism), the gastrointestinal tract (foodborne and infant botulism), and mucus membranes of wounds (wound botulism). Once toxin is absorbed, the circulatory system transports the toxin to peripheral cholinergic synapses, primarily targeting neuromuscular junctions (Simpson, 2004). The toxin binds irreversibly to high-affinity receptors on motor end plates, is internalized into the nerve cell through receptor-mediated endocytosis, and functionally blocks neurotransmitter (acetylcholine) release, causing neuromuscular paralysis. The estimated human dose (assuming 70 kg weight) of type A toxin (extrapolated from non-human primate data) that is lethal to 50% of a population exposed (i.e., the $LD_{50}$) is approximately 0.09–0.15 μg by intravenous administration, 0.7–0.9 μg for inhalation exposure, and 70 μg by way of the oral route (Herrero *et al*., 1967; Scott and Suzuki, 1988; Schantz and Johnson, 1992; Franz *et al*., 1993).

As is the case with most naturally occurring illnesses, exposure to a disease-causing agent would go unnoticed in a bioterrorist attack. The first signs of an outbreak would occur when the hospitals and urgent care medical facilities began receiving victims. The initial diagnosis of botulism is clinical, with confirmatory laboratory findings days away. Neurological signs and symptoms resulting from a toxin-induced blockade of neurotransmission at voluntary motor and cholinergic junctions dominate the clinical manifestation of botulism (Merson and Dowell, 1973; Hughes *et al*., 1981; Wilson *et al*., 1982). Patients with botulism usually present with an acute onset of weakness in muscles innervated by the cranial nerves, leading to diplopia, dysphonia, dysphagia, and dysarthria. In mild cases, no other symptoms may develop. In more severe cases, symmetric weakness progresses in a descending manner, leading frequently to a flaccid paralysis. If the illness is severe enough, the respiratory muscles are paralyzed, leading to ventilatory failure and death unless intubation and mechanical ventilation assistance are initiated. In such cases, antibody therapy is also indicated to inactivate and clear toxin from the circulatory system before it can enter peripheral cholinergic nerve cells and cause further damage to neuromuscular junctions. Patients may also have evidence of autonomic dysfunction including dry mouth, blurred vision, orthostatic hypotension, urinary retention, and constipation. Sensory abnormalities are usually absent, as only motor and autonomic nerves are affected. Similarly, mental function is usually not affected and patients are afebrile. If botulism is a consequence of ingesting improperly preserved foodstuffs contaminated with bacteria and their pre-formed toxins, nausea, abdominal pain, vomiting, and diarrhea may often precede or accompany the neurological abnormalities. Paralysis from botulism can be long-lasting. Mechanical ventilation may be required for two to eight weeks with foodborne botulism, with paralysis lasting as long as seven months (Hughes *et al*., 1981). Symptoms of cranial nerve dysfunction and mild autonomic dysfunction may persist for more than a year (Maroon, 1977; Mann *et al*., 1981; Ehrenreich *et al*., 1989).

It is quite clear from the medical intervention required to assist patients with botulism that a successful bioterrorist attack on large numbers of the public with botulinum neurotoxin would overwhelm the public health system and impact its ability to respond to the victims of such an attack. In 1984, a cult known as the Rajneeshees carried out a successful large-scale biological attack on the citizens of The Dalles town in the U.S. state of Oregon. The plot was unsophisticated, requiring only acquisition and propagation of a common foodborne pathogen (*Salmonella typhimurium* in this case), and using it to contaminate self-service food bars at some 10–12 local restaurants in the area. A total of 751 persons with *Salmonella* gastroenteritis were identified as being associated with eating or working at area restaurants. Fortunately, there were no fatalities and only 6% of those affected required hospitalization (Torok, 1997; Carus, 2000; Miller *et al*., 2001). One has to ask, what the outcome would have been if a colorless, odorless, and tasteless solution of BoNT had been used in lieu of *S. typhimurium*.

*C. botulinum* bacteria are sporulating, obligate anaerobic, Gram-positive bacilli. The spores are ubiquitous and are widely distributed in soil and marine sediments worldwide (Ward, 1967; Smith, 1978; Sugiyama, 1980; Dodds, 1992; Popoff, 1995), and are quite frequently found in the intestinal tract of grazing domestic animals. Under appropriate environmental or laboratory conditions, spores can germinate into vegetative cells and growing vegetative cells will produce toxin. Examples where spores and conditions for promoting their germination and subsequent propagation of toxin-producing bacteria have been found include the improper preservation of foodstuffs (van Ermengen, 1897; Landman, 1904; Leuchs, 1910; Ball *et al*., 1979; Dodds, 1990; O'Mahony, 1990; Hatheway, 1992; Sobel *et al*., 2004; Varma *et al*. 2004), decaying vegetable matter, fish, birds, and other animal carcasses (Smart, 1980; Smart, 1987; Shaffer, 1990; Whitlock, 1997; McLaughlin, 2004), and microbiology laboratories (Lewis and Hill, 1947; Schmidt, 1964; Seigel and Metzger, 1979; Shone, 1995; Malizio, 2000). Because of its seemingly pervasive availability, it would appear to the trained microbiologist that acquisition of a *C. botulinum* strain would not be a limiting factor to the would-be terrorist who seeks to obtain such a strain and who has the expertise and resources to do so. In fact, Internet sites, libraries with scientific journals and books, and bookstores abound with information on how to isolate and culture anaerobic bacteria and specifically, how to produce botulinum neurotoxin. It is a wonder why there has not been a successful bioterrorist attack as yet employing botulinum neurotoxin, keeping in mind the simplest fact, that historically, the major cause of botulism was due to the ingestion of foodstuffs contaminated with *C. botulinum* and pre-formed toxin. The food supply remains our most vulnerable commodity to protect against a botulinum neurotoxin attack.

Botulinum neurotoxin was developed as a biological weapon by several countries, including Japan, Germany, Russia, Iraq, and the United States. The earliest modern use of botulinum neurotoxin as a weapon was by General Shiro Ishii, the head of the Japanese biological warfare command, Unit 731, who admitted to feeding cultures of *C. botulinum* to prisoners with lethal

effect during Japan's occupation of Manchuria in the early 1930s (Hill, 1947). The U.S. first produced botulinum neurotoxin during World War II in response to concerns that Germany had developed the toxin as a weapon to be used against Allied forces. The U.S. produced over 1 million doses of a botulinum toxoid vaccine for the Allied forces preparing to invade Normandy on D-Day (Cochrane, 1947; Bryden, 1989). Because of its lethal potency, the U.S. investigated the potential of botulinum neurotoxin as an offensive biological weapon during the 1940s (Bernstein, 1987; Bernstein, 1990; Franz *et al*., 1997a). The U.S. code name for botulinum neurotoxin was agent X. As noted above, the U.S. offensive biological program on botulinum neurotoxin and all other biological agents ended in 1969–1970 by executive orders from President Richard M. Nixon, and all stockpiles of toxin were destroyed.

Botulinum neurotoxin research, weapons development, and production were a part of the former Soviet Union biological warfare program (United Nations Security Council, 1995; Bozheyeva, 1999). The Soviet Union reportedly tested botulinum-filled weapons at the Soviet site Aralsk-7 on Vozrozhdeniye Island in the Aral Sea (Bozheyeva, 1999; Miller, 1999) and endeavored to use genetic engineering technology to transfer complete toxin genes into other bacteria (Alibek, 1999). Unlike the U.S., the former Soviet Union not only continued but significantly expanded their offensive biological weapons program after signing the BWC treaty in 1972 with ratification in 1975. By signing and ratifying the treaty, the Soviet Union thereby agreed to stop all offensive biological weapons development and destroy existing stockpiles of biological weapons. In April 1992, President Boris Yeltsin publicly declared that his country had continued what is now known to have been a massive offensive biological warfare buildup, which included developing BoNT for weapon use. That same year, the former deputy chief of Biopreparat (the Soviet agency whose primary function was to develop and produce weapons of mass casualties), Colonel Ken Alibek, defected to the U.S. and described the foreboding Soviet biological weapons program in great detail (Alibek, 1999).

Iraq's offensive biological weapons program significantly expanded in 1985, thirteen years after Iraq signed the BWC treaty. In 1995, Iraq admitted to the United Nations Special Commission (UNSCOM) inspection team to having produced 19,000 liters (L) of concentrated botulinum neurotoxin for use in specially designed missiles, bombs, and tank sprayers (United Nations Security Council, 1995; Zilinskas, 1997). Of the 19,000 liters of botulinum neurotoxin concentrate produced between 1989 and 1990 at Al Hakam and Al Manal, 10,000 liters were used to fill 13 Al Hussein SCUD missiles having a 600-km range and 100 400-lb (R-400) bombs (each bomb could hold 85 liters of toxin solution). Biological agents were never used by Iraq during the Gulf wars, and Iraq maintains all stockpiles were either destroyed by Iraq or by UNSCOM inspectors (Blik, 2004).

Aum Shirinkyo, a Japanese cult formed in 1987 by Chizuo Matsumoto (alias Shoko Asahara), obtained religious corporation status from the Tokyo Metropolitan Government in 1989. The cult strived to develop biological weapons after their political party (the Supreme Truth or Shinrito Party) was defeated in the Japanese Diet Election campaign of 1990. Botulinum neurotoxin was one of the first biological agents the cult attempted to produce. Senior followers of the cult obtained soil samples from northern Japan (Hokkaido) with the intent of isolating the *C. botulinum* bacterium. A production facility was constructed at the Aum Shinrikyo compound in the Kamiku-Isshiki village near Mt. Fuji for manufacturing the neurotoxin (Sugishima, 2003). The cult dispersed aerosols of what they thought contained botulinum neurotoxin at multiple sites in downtown Tokyo, Japan, and at U.S. military installations in Japan on at least three occasions between 1990 and 1995. These attacks all failed as there were no reports of a botulism outbreak in Japan during this period. Additionally, shortly preceding the sarin attack in the Tokyo subway, three attaché cases containing portable disseminating devices generating water vapor were found in the subway station. The cult's perpetrator for these devices said in testimony at Shoko Asahara's trial in 1996 that he believed those cases contained botulinum neurotoxin. It was reported that botulinum neurotoxin had not been detected in those devices. In 1995, the cult had some 50,000 followers worldwide and an estimated US$1 billion in financial resources (Sugishima, 2003). Thus, the cult had the resources to develop biological toxins for use as weapons, the intent to do so, and although they had no specialist in the development of biological weapons, among their followers were microbiologists, medical doctors, and other scientists. It is not completely clear why the biological assaults failed, but information placed in evidence at Asahara's trial indicated that the cult's scientists had difficulty overcoming the technical barriers in isolating and cultivating *C. botulinum* and may never have been successful in doing so (Sugishima, 2003).

## *Staphylococcus aureus* enterotoxins

*Staphylococcus aureus* bacteria are facultative anaerobic, non-sporulating, Gram-positive cocci. They pro-

duce a host of exotoxins that contribute to their ability to colonize and cause disease in mammalian hosts (Johnson *et al*., 1992; Monday and Bohach, 1999; Dinges *et al*., 2000). Staphylococcal enterotoxins are 23 to 29 kDa polypeptides in the bacterial superantigen family (Ulrich *et al*., 1995; Ulrich, 2000) and comprise at least 15 antigenically different enterotoxin serotypes (Balaban and Rasooly, 2000; Krakauer and Stiles, 2003). They are the most frequent cause of food poisoning (Holmberg and Blake, 1984; Tranter, 1990; Granum and Brynestad, 1999) and as little as 100–200 ng can cause emesis in humans (Jablonski and Bohach, 1997). Staphylococcal superantigens cause disease by binding to monocytes at major histocompatibility complex (MHC) type II receptors rather than the usual antigen-binding receptors (Johnson *et al*., 1992; Monday and Bohach, 1999). Conventional antigens are normally taken up and proteolytically processed by antigen-presenting cells (APC). They are then presented for recognition by multiple elements in the T cell receptor (TCR) in the binding cleft of MHC class II receptors. SE and other superantigens are not processed by APCs, but associate directly with MHC class II and the TCR complex outside of the typical binding cleft. This induces a cascade of pro-inflammatory cytokines (such as tumor necrosis factor, interferon, interleukin-1, and interleukin-2), with recruitment of other immune effector cells, and relatively little activation of counter-regulatory feedback loops. Released cytokines are thought to mediate many of the toxic effects of the enterotoxins.

During the 1960s, as part of its biological warfare program, the United States investigated staphylococcal enterotoxin B (then code-named PG) for its potential use as an incapacitating agent (Ulrich *et al*., 1997). Staphylococcal enterotoxin B was especially attractive as a biological agent because much lower quantities were needed to produce the desired incapacitating effect than were required with synthetic chemicals. The estimated human dose that is incapacitating for 50% of the population exposed (also called the effective dose [$ED_{50}$]) is 0.0004 μg/kg, and the estimated human dose that is lethal for 50% of the population exposed ($LD_{50}$) is estimated to be 0.02 μg/kg, both by the inhalation route (Ulrich *et al*., 1997; Franz and Zajtchuk, 1997). No data exist on the $LD_{50}$ and $ED_{50}$ in humans by other routes of exposure (Rusnak *et al*., 2004).

Like other biological threat agents, diagnosis of staphylococcal enterotoxin B intoxication is initially based on clinical and epidemiological findings. Clinical symptoms from intoxication with staphylococcal enterotoxins include non-specific flu-like symptoms, including fever, chills, headache, myalgia, and varying degrees of prostration. Additional symptoms are specific to the exposure route (Greenfield, 2002; Franz *et al*., 1997b; Zapor and Fishbain, 2004; Rusnak *et al*., 2004). The incubation period from oral intoxication is one to four hours after oral ingestion. Usual symptoms include nausea, vomiting, abdominal cramping, and diarrhea. Symptoms typically last up to 20 hours. With severe intoxication, there can be profound dehydration due to loss of fluids, shock, respiratory failure, and cardiovascular collapse. Approximately 15% of patients require hospitalization, with a 5% fatality rate, usually in the very young or the elderly. Treatment for staphylococcal enterotoxin B intoxication is supportive care, as no specific approved antitoxin or vaccine exists; however, experimental vaccines are under development.

Staphylococcal enterotoxin B inhalation exposure presents with different symptoms than seen with oral ingestion. Inhalation causes respiratory signs, including a non-productive cough, chest pain, and dyspnea. In severe cases, there may be pulmonary edema, respiratory distress syndrome, shock, and death (Mattix *et al*., 1995; Ulrich *et al*., 1997). Gastrointestinal signs may also be seen after an aerosol exposure and are postulated to result from secondary oral ingestion of staphylococcal enterotoxin B concomitant with the inhalation exposure. Recently, a review of 16 occupational exposures to staphylococcal enterotoxin B from 1989 to 2002 at the United States Army Medical Research Institute of Infectious Diseases was reported (Rusnak *et al*., 2004). Conjunctivitis with localized cutaneous swelling occurred in three persons within one to six hours after exposure to staphylococcal enterotoxin B; two of these persons also had gastrointestinal symptoms, which suggests that such symptoms occurred as a result of exposure by an indirect cutaneous or ocular route. This has been the first report of conjunctivitis and local facial swelling due to ocular exposure from staphylococcal enterotoxin B.

## *Clostridium perfringens* epsilon toxin

*Clostridium perfringens* are sporulating, anaerobic, Gram-positive bacilli and, like other clostridial species, their spores are ubiquitous. *C. perfringens* was one of the pathogenic bacteria manufactured and studied by Iraq as part of its biological warfare program (Zilinskas, 1997). *C. perfringens* strains (types A, B, C, D, and E) elicit toxins that cause gas gangrene in humans, necrotizing enteritis in humans and animals, and enterotoxemias in animals. *C. perfringens* type A produces a 35-kDa enterotoxin (CPE) that is responsible for the symptoms of common human food poisoning and acts by forming pores in plasma membranes,

allowing electrolytes and fluids to escape (Skjelkvale and Uemura, 1977; Olsen *et al*., 2000; Adak *et al*., 2002). *C. perfringens* types B and D produce epsilon toxin within the gut of infected animals, mainly lambs and goats, and cause fatal veterinary enterotoxemias after the toxin has been absorbed across the mucosal barrier of their gastrointestinal tract (Songer, 1996; Smedley *et al*., 2004). Although *C. perfringens* epsilon toxin is a Category B biothreat agent, there have been no reported cases of human disease with this toxin. Epsilon toxin (33 kDa) is synthesized as a relatively inactive precursor toxin that becomes activated by a post-translational processing step in which 13 N-terminal and 22 C-terminal amino acid residues are proteolytically removed (Hunter *et al*., 1992; Miyata *et al*., 2001). Epsilon toxin has been shown to form oligomeric pores in intestinal and extraintestinal target tissues (Petit *et al*., 2001; Miyata *et al*., 2001; Miyata *et al*., 2002; Petit *et al*., 2003). Studies investigating the distribution and localization of the toxin after systemic administration in mice found the toxin to localize primarily in nasal cavity, spinal cord, brain, and kidneys (Tamai *et al*., 2003), supporting the belief that the toxin's primary mechanism for toxicity is to cause edema and hemorrhage in various organs such as brain, kidneys, and lungs (Petit *et al*., 2003). Neurological disorders, including convulsions and opisthotonos, have been noted in rodents after epsilon toxin intoxication, signs believed to result from damage to brain vasculature and direct effects on hippocampal cells (Miyamoto *et al*., 1998; Miyamoto *et al*., 2000). The lethal dose of epsilon toxin for rodents is 100 ng/kg (Franz, 1997), and a comparable parental dose for a 70 kg human would be 7 μg. It is thought that a biothreat delivery would be via the aerosol/inhalation route, leading to pulmonary edema followed by central nervous system, renal, and cardiovascular damage (Greenfiled *et al*., 2002). There are no antidotes for this toxin.

## CONCLUSION

Before the terrorist attacks on the World Trade Center, the anthrax letters, and the rash of suicide bombings in the Middle East, there had been numerous warnings regarding the potential for terrorism and the realization that such aggression could be perpetrated with biological weapons (Dando, 1994; Danzig, 1997; Holloway, 1997; Takafuji *et al*.,1997; Falkenrath *et al*., 1998; Henderson, 1999; Kortepeter and Parker, 1999; Wilkening, 1999; Gurr and Cole, 2000). In fact, the U.S. Congress developed a comprehensive legal framework to prevent the illegitimate use of toxins and infectious agents and signed into law the Biological Weapons Act of 1989 and the Anti-Terrorism and Effective Death Penalty Act of 1996 (Ferguson, 1997). Of course, laws are usually not deterrents to bioterrorists and do not prevent them from committing acts of violence. Enactment of laws, however, does reflect the concern of the government towards the use of biological agents in acts of bioterrorism and biocrimes. Much of the attention in the1990s over bioterrorism was due to the violations of the 1972 BWC accord and the continued offensive biological warfare programs in the former Soviet Union and Iraq. The economic and political collapse of the Soviet Union heightened these concerns, including fears that scientists working in the Soviet biological warfare program would travel to Iraq, Iran, Syria, Libya, North Korea, and other countries to sell Soviet-produced biothreat agents (i.e., toxins, bacterial, and viral strains), as well as their scientific expertise (Alibek, 1999).

In response, a Trilateral Agreement between the U.S., the U.K., and Russia was signed in 1992, which was intended to build confidence that Russia would dismantle the former Soviet Union's offensive biological weapons program (Caudle, 1997). Additionally, it committed Russia to opening suspect facilities for inspection, converting biological warfare facilities to more productive uses, and ending all biological weapons projects except for defensive purposes. Before the anthrax letters in 2001, the U.S. government had begun preparing for a potential bioterrorist attack (Tucker, 1997; Stephenson, 1997). After the anthrax letters, the response was accelerated (Lane, 2001). Federal bioterrorism expenditures increased from $414 million in FY2001 to over $5.5 billion in FY2004 and are expected to increase by another $2.1 billion in FY2005. Between FY2001 and FY2005, it is estimated that the U.S. government will spend $22.1 billion on civilian biodefense (Schuler, 2004). Project BioShield, signed into law by President Bush on July 21, 2004, provided $6 billion over the next 10 years for the development and production of vaccines and drugs to be used in the event of another bioterrorist attack. The Presidential Directive "Biodefense for the 21st Century" signed April 28, 2004 by President Bush, reaffirmed the nation's commitment to protect its homeland and U.S. global interests against bioattacks. Historically, there have been relatively few biowarfare and bioterrorist attacks worldwide. However, as noted above, they do occur. Pathogens and toxins exist in nature and are potentially available to those who have a strong desire to acquire and employ them as weapons.

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

## REFERENCES


Adak, G.K, Long, S.M, and O'Brien, S.J. (2002). Trends in indigenous foodborne disease and deaths, England and Wales:1992 to 2000. *Gut* **51,** 832–841

Alibek, K. and Handleman, S. (1999). *Biohazard*. Random House, New York, N.Y.

Arnon, S.A., R. Schecter, T.V. Inglesby, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A.D. Fine, J. Hauer, M. Layton, S. Lillibridge, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, D.L. Swerdlow, and K. Tonat, (2001). Botulinum toxin as a biological weapon. *JAMA* **285,** 1059–1070.

Aureli, P., Fenicia, L., Pasolini, B., Gianfranceschi, M., McCroskey, L.M. and Hatheway, C.L. (1986). Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy. *J. Infect. Dis*. **154,** 207–211.

Balaban, N. and Rasooly, A. (2000) Staphylococcal enterotoxins. *Int. J. Food Microbiol*. **61,** 1–10.

Ball, A.P., Hopkinson, R.B., Farrell, I.D., Hutchison, J.G.P., Paul, R., Watson, R.D.S., Page, A.J., Parker, R., Edwards, C.W., Snow, M., Scott, D.K., Leone-Ganado, A., Hastings, A., Ghosh, A.C. and Gilbert, R.J. (1979). Human botulism caused by *Clostridium botulinum* type E: The Birmingham outbreak. *Quart. J. Med*. **48,** 473–491.

Barnaby, W. (1997). *The Plague Makers: The Secret World of Biological Warfare*. pp. 5–20. Satin Publications Limited, London.

Bernstein, B.J. (1987). The birth of the U.S. biological warfare program. *Sci Am*. **256,** 116–121.

Bernstein, B.J. (1990). Origins of the U.S. biological warfare program. In: *Preventing a Biological Arms Race* (ed. S. Wright), Chapter 1, pp. 9–25. The MIT Press, Cambridge, MA.

Blik, H. (2004). *Disarming Iraq*. pp. 67, 256–257. Pantheon Books, New York, N.Y.

Bozheyeva, G., Kunakbayev, Y., Yeleukenov, D. (1999). Former Soviet Biological Weapons Facilities in Kazakhstan: Past, Present, and Future. Occasional paper No. 1, pp. 1–20. Center for Nonproliferation Studies, Monterey Institute of International Studies, Monterey, CA.

Bryden, J. (1989). *Deadly Allies: Canada's Secret War, 1937–1947*. McClelland & Stewart, Toronto, Ontario.

Butler, R. (2000). *The Greatest Threat: Iraq, Weapons of Mass Destruction and the Crisis of Global Security*. pp. 86–88. Public Affairs, New York, N.Y.

Cartwright F.F. (1974). *Disease and History*. pp. 124–125. New American Library. New York, N.Y.

Carus, W.S. (2000). The Rajneeshees (1984). In: *Toxic Terror: Assessing Terrorist Use of Chemical and Biological Weapons* (ed. J.B. Tucker), Chapter 8, pp. 115–137. MIT Press, Cambridge, MA.

Carus, W.S. (2001). *Working Paper Bioterrorism and Biocrimes: The Illicit Use of Biological Agents Since 1900*. Center for Counterproliferation Research, National Defense Univeristy, Washington, D.C.

Caudle III, L.C. (1997). The biological warfare threat. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji and D.R. Franz), Chapter 21, pp. 451–466. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Centers for Disease Control and Prevention (1998). *Botulism in the United States, 1899–1998. Handbook for epidemiologists, clinicians, and laboratory workers*. U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia.

Christopher, G.W., Cieslak, T.J., Pavlin, J.A. and Eitzen, E.M. (1997). Biological warfare: a historical perspective. *JAMA* **278,** 412–417.

Cochrane, R.C. (1947). Biological Warfare Research in the United States. In: *History of the Chemical Warfare Service in World War II (01 July 1940–15 August 1945)*. Vol 2. Historical Section, Plans, Training and Intelligence Division, Office of Chief, Chemical Corps, U.S. Department of the Army. Unclassified. Archived at the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD.

Cole, L.A. (1988). *Clouds of Secrecy: The Army's Germ Warfare Tests Over Populated Areas*. pp. 11–12. Rowman and Littlefield, Totowa, N.J.

Dando, M. (1994). *Biological Warfare in the 21st Century*. pp. 1–15. Brassey's (UK), London.

Danzig, R. and Berkowsky, P.B. (1997). Why should we be concerned about biological warfare? *JAMA*. **278,** 431–2.

de Paiva, A., Meunier, F.A., Molgo, J., Aoki, K.R. and J.O. Dolly (1999). Functional repair of motor endplates after botulinum neurotoxin type A poisoning: biphasic switch of synaptic activity between nerve sprouts and their parent terminals. *Proc Natl Acad Sci USA* **96,** 3200–5.

Derbes, V.J. (1966). De Mussis and the great plague of 1348: A forgotten episode of bacteriological warfare. *JAMA*. **196,** 179–182.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000) Exotoxins from *Staphylococcus aureus. Microbiol. Rev*. **13,** 16–34.

Dodds, K.L. (1990). Restaurant-associated botulism outbreaks in North America. *Food Control*. **1,** 139–141.

Dodds, K.L. (1992). *Clostridium botulinum* in the environment. In: *Clostridium Botulinum—Ecology and Control in Foods* (eds. A.H.W. Hauschild and K.L. Dodds), pp. 21–51. Marcel Dekker Inc., New York, N.Y.

Dolman, C.E. and Murakami, L. (1961). *Clostridium botulinum* type F with recent observations on other types. *J Infect Dis*. **109,** 107–128.

Ehrenreich, H., Garner, C.G. and Witt, T.N. (1989). Complete bilateral internal ophthalmoplegia as sole clinical sign of botulism: confirmation of diagnosis by single fibre electromyography. *J. Neurol*. **236,** 243–5.

Eitzen, E.M. and Takafuji, E.T. (1997). Historical Overview of Biologtical Warfare. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji, and D.R. Franz). Chapter 18, pp. 415–423. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Eleopra. R., Tugnoli, V., Quatrale, R., Rossetto, O., Montecucco, C. and De Grandis, D. (2002). Botulinum neurotoxin serotypes C and E: Clinical trials. In: *Scientific and Therapeutic Aspects of Botulinum Toxin* (eds. M.E. Brin, M.Hallett, and J.Jankovic). Chapter 42, pp. 441–450. Lippincott Williams & Wilkins, New York, N.Y.

Falkenrath, R.A., Newman, R.D. and Thayer, B.A. (1998). *America's Achilles Heel: Nuclear, Biological, and Chemical Terrorism and Covert Attack*. Chapters 3 and 4, pp. 167–260. The MIT Press, Cambridge, MA.

Ferguson, J.R. (1997). Biological weapons and the U.S. law. *JAMA* **278,** 357–360.

Fern, E.A. (2000) Biological warfare in the eighteenth-century North America: beyond Jeffrey Amherst. *J. Am. Hist*. **86,** 1552–1580.

Foran, P., Mohammed, N., Lisk, G., Nagwaney, S., Lawrence, G., Johnson, E., Smith, L. A., Aoki, R. and J. O. Dolly (2003). Evaluation of the therapeutic usefulness of botulinum neurotoxins B, C1, E, and F compared to the long-lasting type A: basis for distinct durations of inhibition of exocytosis in central neurons. *J. Biol. Chem* **278,** 1363–1371.

Franz, D.R., Pitt, L.M., Clayton, M.A., Hanes, M.A. and Rose, K.J. (1993). Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In: *Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects* (ed. B.R. DasGupta) pp. 473–476. Plenum Press, New York, N.Y.

Franz, D.R. (1997). Defense against toxin weapons. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji and D.R. Franz), Chapter 30, pp. 603–619. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Franz, D.R., Parrott, C.D. and Takafuji, E.T. (1997a). The U.S. biological warfare and biological defense programs. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji, and D.R. Franz), Chapter 19, pp. 425–436. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Franz, D.R., Jahrling, P.B., Friedlander, A.M., McClain, D.J., Hoover, D.L., Byrne, W.R., Pavlin, J.A., Christopher, G.W. and Eitzen, E.M. (1997b). Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* **278,** 399–411.

Franz, D.R. and Zajtchuk, (2000). Biological terrorism: understanding the threat, preparation, and medical response. In: *Disease-a-Month* (ed. E.E. Brueschke), Vol. 46, pp. 125–192. Mosby, Inc., Linn, MI.

Geissler, E. (1986). Introduction. In: *Biological and Toxin Weapons Today*. Chapter 1, pp. 1–20. Oxford University Press, Stockholm International Peace Research Institute, Oxford, England.

Gill, D. M. (1982). Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.* **46,** 86–94.

Gold, H. (1996). *Unit 731 Testimony*. YENBOOKS, Singapore.

Granum, P.E. and Brynestad, S. (1999). Bacterial toxins as food poisons. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer). Chapter 38, pp. 669–681. Academic Press, London.

Greenfield, R.A., Brown, B.R., Hutchins, J.B., Iandolo, J.J., Jackson, R., Slater, L.N. and Bronze, M.S. (2002). Microbiological, biological, and chemical weapons of warfare and terrorism. *Am. J. Med. Sci*. **323,** 326–340.

Gunnison, J.B. and Meyer, K.F. (1930). Susceptibility of monkeys, goats, and small animals to oral administration of botulinum toxin types B, C, and D. *J Infect Dis*. **46,** 335–340.

Gurr, N. and Cole, B. (2000). *The New Face of Terrorism: Threat from Weapons of Mass Destruction*. pp. 1–21. I.B. Tauris Publishers, London.

Hall, J.D., McCroskey, L.M., Pincomb, B.J., and Hatheway, C.L. (1985). Isolation of an organism resembling *Clostridium baratii* which produces type F botulinal toxin from an infant with botulism. *J Clin Microbiol*. **21,** 654–655.

Harris, S.H. (1994). *Factories of Death: Japanese Biological Warfare, 1932–45, and the American Cover-up*. Routledge, New York, N.Y.

Harris, S.H. (2003). Japanese biomedical experimentation during the World-War-II era. In: *Military Medical Ethics, Volume II* (eds. T.E. Beam and L.R Sparacino). Chapter 16, pp. 463–506. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Harris, R. and Paxman, J. (1982). *A Higher Form of Killing*. pp. 76–79, 153. Hill and Wang, New York, N.Y.

Hatheway, C. L. (1992). *Clostridium botulinum* and other clostridia that produce botulinum neurotoxins. In: *Clostridium Botulinum-ecology and Control in Foods* (eds. A.H.W. Hauschild and K.L. Dodds), pp. 3–10. Marcel Dekker Inc., New York, N.Y.

Henderson, D.A. (1999). The looming threat of bioterrorism. *Science* **283,** 1279–82.

Herrero, B.A., Ecklung, A.E., Streett, C.S., Ford, D.F. and and J.K. King (1967). Experimental botulism in monkeys—a clinical pathological study. *Exp Mol Pathol*, **6,** 84–95.

Hill, E.V. (1947). Botulism. In: *Summary Report on B. W. Investigations*. Memorandum to Alden C. Waitt, Chief Chemical Corps, United States Army, December 12, 1947; tab D. Archived at the U.S. Library of Congress, Washington, D.C.

Holloway, H.C., Norwood, A.E., Fullerton, C.S., Engel Jr. and Ursano, R.J. (1997). The threat of biological weapons: prophylaxis and mitigation of psychological and social consequences. *JAMA* **278,** 425–427.

Holmberg, S.D. and Blake, P.A. (1984). Staphylococcal food poisoning in the United States. *JAMA* **251,** 487–489.

Hughes, J.M., Blumenthal, J.R., Merson, M.H., Lombard, G.L., Dowell, Jr. V.R. and E.J. Gangarosa (1981). Clinical features of types A and B food-borne botulism. *Ann. Intern. Med*. **95,** 442–5.

Hunter, S. E., Clarke, I.N., Kelly, D.C. and Titball, R.W. (1992). Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. *Infect. Immun*. **60,** 102–110.

Jablonski, K.M. and Bohach, G.A. (1997). *Staphylococcus aureus*. In: *Food Microbiology: Fundamentals and Frontiers* (eds. M, Doyle, L. Beuchat and T. Montville), pp. 327–336. ASM Press, Washington, D.C.

Johnson, H.M., Russell, J.K. and Pontzer, C.H. (1992). Superantigens in human disease. *Sci Am*. **266,** 92–101.

Kolavic, S.A., Kimura, A., Simmons, S.L., Slutsker, L., Barth, S. and Haley, C.E. (1997). An outbreak of *Shigella dysenteriae* type 2 among laboratory workers due to intentional food contamination. *JAMA* **278,** 396–398.

Kortepeter, M. and Parker, G.W. (1999). Potential biological weapons threats. *Emerg. Infect. Dis*. **5,** 523–527.

Krakauer T. and Stiles, B.G. (2000). Staphylococcal enterotoxins, toxic shock syndrome toxin-1, and streptococcal pyrogenic exotoxins: some basic biology of bacterial superantigens. *Recent Res. Develop. Infect. Immun*. **1,** 1–27.

Kupperman, R.H. and Smith, D.M. (1993). Coping with biological terrorism. In: *Biological Weapons: Weapons of the Future?* (ed. B. Roberts), pp. 35–46. The Center for Strategic and International Studies, Washington, D.C.

Lamanna, C. (1959). The most poisonous poison. *Science* **130,** 763–772.

Landmann, G. (1904). Ueber die Ursache der darmstadter Bohnenvergiftung. *Hyg. Rundschau*. **14,** 449–452.

Lane, H.C., LaMontagne, J. and Fauci, A.S. (2001). A clear and present danger. *Nature Med*. **7,** 1271–1273.

Leuchs, J. (1910). Beitrage zur Kenntnis des Toxins and Antitoxins des *Bacillus botulinus*. *Z. Hyg. Infektionskrankh* **65,** 55–84.

Lewis, K.H. and Hill, E.V. (1947). Practical media and control measurements for producing highly toxic cultures of *Clostridium botulinum*, type A. *J. Bacteriol*. **53,** 213–230.

Malizio, C.J., Goodnough, M.C. and Johnson, E.A. (2000). Purification of *Clostridium botulinum* type A neurotoxin. In: *Methods in Molecular Biology, Bacterial Toxins: Methods and Protocols* (ed. O. Holst), Vol. 145, pp. 27–39. Humana Press, Totowa, New Jersey.

Mann, J.M., Martin, S., Hoffman, R. and Marrazzo, S. (1981). Patient recovery from type A botulism: morbidity assessment following a large outbreak. *Am. J. Public Health* **71,** 266–9.

Maroon, J.C. (1977). Late effects of botulinum intoxication. *JAMA* **238,** 129.

Mattix, M.E., Hunt, R.E., Wilhelmson, C.L., Johnson, A.J. and Baze, W.B. (1995). Aerosolized staphylococcal enterotoxin B-induced pulmonary lesions in rhesus monkeys (*Macaca mulatta*). *Toxicol Pathol*. **23,** 262–8.

McLaughlin, J.B., Sobel, J., Lynn, T., Funk, E. and Middaugh, J.P. (2004). Botulism type E outbreak associated with eating a beached whale, Alaska. *Emerg. Infec. Dis*. **10,** 1685–1687.

Mee, C. (1990). How a mysterious disease laid low Europe's masses. *Smithsonian*. **20,** 66–79.

Merson, M.H. and Dowell, V. R., Jr. (1973). Epidemiologic, clinical, and laboratory aspects of wound botulism. *N. Engl. J. Med*. **289,** 1105–10.

Meselson, M., Guillemin, J., Hugh-Jones, M., Langmuir, A., Popova, I. and Yampolskaya, O. (1994). The Sverdlovsk anthrax outbreak of 1979. *Science* **266,** 1202–1208.

Middlebrook, J.L. and Franz, D.R. (1997) Botulinum toxins. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji, and D.R. Franz), Chapter 33, pp.643–654. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Miller, J. (1999) At bleak Asian site, killer germs survive. *New York Times*. June 2, 1999:A1, A10.

Miller, J. Engelberg, S. and Broad, W. (2001). *Germs: Biological Weapons and America's Secret War*, pp. 15–33. Simon and Schuster, New York, N.Y.

Miyata, S., Matsushita, O., Minami, J., Katayama, S., Shimamoto, S., and Okabe, A. (2001). Cleavage of a C terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon toxin in the synaptosomal membrane. *J. Biol. Chem*. **276,** 13778–13783.

Miyata, S., Minami, J., Tamai, E., Matsushita, O., Shimamoto, S. and Okabe, A. (2002). *Clostridium perfringens* epsilon toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. *J. Biol. Chem*. **277,** 39463–39468.

Miyamoto, O., Minami, J., Toyoshima, T., Nakamura, T., Masada, T., Nagao, S., Negi, T., Itano, T. and Okabe, A. (1998). Neurotoxicity of *Clostridium perfringens* epsilon-toxin for the rat hippocampus via the glutamatergic system. *Infect. Immun*. **66,** 2501–2508.

Miyamoto, O., Sumitani, K., Nakamura, T., Yamagami, S., Miyata, S., Itano, T., Negi, T. and Okabe, A. (2000). *Clostridium perfringens* epsilon toxin causes excessive release of glutamate in the mouse hippocampus. *FEMS Microbiol. Lett*. **189,** 109–113.

Monday, S.R. and Bohach, G.A. (1999). Properties of *Staphylococcus aureus* enterotoxins and toxic shock syndrome toxin-1. In: *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds. J.E. Alouf and J.H. Freer), Chapter 33, pp. 589–610. Academic Press, London.

Ohishi, I. (1984). Oral toxicities of *Clostridium botulinum* type A and B toxins from different strains. *Infect. Immun*. **43,** 487–490.

Olsen, S.J., MacKinnon, L.C., Goulding, J.S., Bean, N.H. and Slutsker, L. (2000). Surveillance for foodborne-disease outbreaks—United States, 1993–1997. *MMWR CDC Surveill Summ* **49,** 1–62.

O'Mahony, M. Mitchell, E., Gilbert, R.J., Hutchinson, D.N., Begg, N.T., Rodhouse, J.C. and Morris, J.E. (1990). An outbreak of foodborne botulism associated with contaminated hazelnut yoghurt. *Epidemiol Infect*. **104,** 389–95.

Parkman, F. (1969). *The Conspiracy of Pontiac*. Vol 2. pp. 44–46. Little Brown, Boston, Mass.

Petit, L., Maier, E., Gibert, M., Popoff, M.R. and Benz, R. (2001). *Clostridium perfringens* epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. *J. Biol. Chem*. **276,** 15736–15740.

Petit, L., Gibert, M., Gourch, A., Bens, M., Vandewalle, A. and Popoff, M.R. (2003). *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. *Cell. Microbiol*. **5,** 155–164.

Popoff, M.R. (1995). Ecology of neurotoxigenic strains of clostridia. In: *Current Topics in Microbiology: Clostridial Neurotoxins The Molecular Pathigenesis of Tetanus and Botulism* (ed. C. Montecucco), Vol. 195, pp. 1–29. Springer-Verlag, Berlin.

Poupard, J.A., Miller, L.A. and Granshaw, L. (1989). The use of smallpox as a biological weapon in the French and Indian War of 1763. *ASM News* **55,** 122–124.

Rotz, L.D., Khan, A.S., Lillibridge, S.R., Ostroff, S.M. and Hughes, J.M. (2002). Public health assessment of potential biological terrorism agents. *JAMA* **8,** 225–230.

Rusnak, J.M., Kortepeter, M., Ulrich, R., Poli, M. and Boudreau, E. (2004). Laboratory exposures to Staphylococcal enterotoxin B. *Emerg. Infect. Dis*. **10,** 1544–1549.

Schantz, E.J. and Johnson, E.A. (1992). Properties and use of botulinum toxin and other microbial neurotoxins in medicine. *Microbiol Rev*. **56,** 80–99.

Schmidt, C.F. (1964). Spores of *C. botulinum*: formation, resistance, germination. In: *Botulism: Proceedings of a Symposium* (eds. K.H. Lewis and K. Cassel Jr.), pp. 69–82. U.S. Department of Health, Education, and Welfare, Public Health Service, Cincinnati, Ohio.

Schuler, A. (2004). Billions for biodefense: federal agency biodefense funding, FY2001-FY2005. *Biosecurity Bioterrorism* **2,** 86–96.

Scott, A.B. and Suzuki, D. (1988). Systemic toxicity of botulinum toxin by intramuscular injection in the monkey. *Mov. Disord*. 333–5.

Shaffer, N., Wainwright, R.B., Middaugh, J.P. and Tauxe, R.V. (1990). Botulism among Alaska natives. The role of changing food preparation and consumption practices. *West J. Med*. **153,** 390–3.

Shaffer, D., Armstrong, G., Higgins, K., Honig, P., Coyne, P., Boxwell, D., Beitz, J., Leissa, B. and Murphy, D. (2003). Increased U.S. prescription trends associated with the CDC *Bacillus anthracis* antimicrobial postexposure prophylaxis campaign. *Pharmacoepidemiol. Drug. Saf*. **12,** 177–182.

Shone, C.C. and Tranter, H.S. (1995). Growth of clostridia and preparation of their neurotoxins. In: *Current Topics in Microbiology: Clostridial Neurotoxins: The Molecular Pathigenesis of Tetanus and Botulism* (ed. C. Montecucco), Vol. 195, pp. 143–160. Springer-Verlag, Berlin.

Siegel, L.S. and Metzger, J.F. (1979). Toxin production by *Clostridium botulinum* type A under various fermentation conditions. *Appl. Environ. Microbiol*. **38,** 606–611.

Simpson, L.L. (2004). Identification of the major steps in botulinum toxin action. *Ann. Rev. Pharmacol. Toxicol*. **44,** 167–193.

Skjelkvale, R. and Uemura, T. (1977). Experimental diarrhea in human volunteers following oral administration of *Clostridium perfringens* enterotoxin. *J. Appl. Bacteriol*. **43,** 281–286.

Smart, J.L., Roberts, T.A., McCullagh, K.G., Lucke, V.M. and Pearson, H. (1980). An outbreak of type C botulism in captive monkeys. *Vet. Rec*. **107,** 445–446.

Smart, J.L., Jones, T.O., Clegg, F.G. and McMurray, M.J. (1987). Poultry waste associated type C botulism in cattle. *Epidemiol. Inf*. **98,** 73–79.

Smedley III, J.G., Fisher, D.J., Sayeed, S., Chakrabarti, G. and McClane, B.A. (2004). The enteric toxins of Clostridium perfringens. *Rev. Physiol. Biochem. Pharmacol*. **152,** 183–204.

Smith, L.D.S. (1978). The occurence of *Clostridium botulinum* and *Clostridium tetani* in the soil of the United States. *Health Lab Sci*. **15,** 74–80.

Sobel, J., Tucker, N., Sulka, A., McLaughlin, J. and Maslanka, S. (2004) Foodborne botulism in the United States, 1900–2000. *Emerg. Infect. Dis*. **10,** 1606–1611.

Songer, J.G. (1996). Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev*. **9,** 216–234.

Stearn, E.W. and Stearn, A.E. (1945). *The Effects of Smallpox on the Destiny of the Amerindians*. pp. 44–45. Bruce Humphries, Boston, MA.

Stephenson, J. (1997). Pentagon-funded research takes aim at agents of biological warfare. *JAMA* **278,** 373–375.

Sugishima, M. (2003) Aum Shinrikyo and the Japanese law on bioterrorism. *Prehosp. Disas. Med*. **18,** 179–183.

Sugiyama, H. (1980). *Clostridium botulinum* neurotoxin. *Microbiol Rev*. **44,** 419–48.

Takafuji, E.T., Johnson-Winegar, A. and Zajtchuk, R. (1997). Medical challenges in chemical and biological defense for the 21st century. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then*

*Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji and D.R. Franz), Chapter 35, pp. 677–685. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

Tamai, E., Ishida, T., Miyata, S., Matsushita, O., Suda, H., Kobayashi, S., Sonobe, H. and Okabe, A. (2003). Accumulation of *Clostridium perfringens* epsilon toxin in the mouse kidney and its possible biological significance. *Infect. Immun*. **71,** 5371–5375.

Torok, T.J., Tauxe, R.V., Wise, R.P., Livengood, J.R., Sokolow, R., Mauvais, S., Birkness, K.A., Skeels, M.R., Horan, J.M. and Foster, L.R. (1997). A large community outbreak of Salmonellosis caused by intentional contamination of restaurant salad bars. *JAMA* **278,** 389–395.

Tranter, H.S. (1990). Foodborne staphylococcal illness. *Lancet* **336,** 1044–1046.

Tucker, J. (1997). National response and medical services response to incidents of chemical and biological terrorism. *JAMA* **278,** 362–368.

Ulrich, R.G. (2000). Evolving superantigens of *Staphylococcus aureus. FEMS Immunol. Med. Microbiol*. **27,** 1–7.

Ulrich, R.G., Bavari, S. and Olson, M.A. (1995). Bacterial superantigens in human disease: structure, function, and diversity. *Trends Microbiol*. **3,** 463–468.

Ulrich, R.G., Sidell, S., Taylor, T.J., Wilhelmsen, C.L. and Franz, D.R. (1997). Staphylococcal enterotoxin B and related pyrogenic toxins. In: *Textbook of Military Medicine, Part I: Warfare, Weaponry, and then Casualty: Medical Aspects of Chemical and Biological Warfare* (eds. F.R. Slidell, E.T. Takafuji, and D.R. Franz), Chapter 31, pp.621–630. Borden Institute, Walter Reed Army Medical Center, Washington, D.C.

United Nations Security Council. (1995). *Tenth Report of the Executive Chairman of the Special Commission Established by the Secretary-General Pursuant to Paragraph 9(b)(I) of Security Council Resolution 687 (1991), and Paragraph 3 of Resolution 699 (1991) on the Activities of the Special Commission*. S/1995/1038. United Nations Security Council, New York, N.Y.

van Ermengen, E. (1897). Ueber einen neuren anaeroben *Bacillus* und seine beziehungen zum botilismus. *Z. Hyg. Infektionskrankh*. **26,** 1–56.

Varma, J.K., Katsitadze, G., Moiscrafishvili, M., Zardiashvili, T., Chokheli, N., Jhorjholiani, E., Chubinidze, M., Kukhalashvili, T., Kkmaladze, I., Chakvetadze, N., Imnagze, P. and Sobel, J. (2004) Foodborne botulism in the Republic of Georgia. *Emerg. Infect. Dis*. **10,** 1601–1605.

Ward, B.Q., Carroll, B.J., Garrett, E.S. and Reese, G.B. (1967). Survey of the U.S. Gulf coast for the presence of *Clostridium botulinum. Appl Microbiol*. **15,** 629–36.

Warner, R. (translator) (1972). *Thucydides, The History of the Peloponnesian War, 431 B.C.E.* Viking Press, New York, N.Y.

Wheelis, M. (1999). Biological warfare before 1914. In: *Biological and Toxin Weapons: Research, Development, and Use from the Middle Ages to 1945* (eds. E. Geissler and J.E. van Courtland Moon), pp. 21–24. Oxford University Press for the Stockholm International Peace Research Institute, Oxford, England.

Whitlock, R.H. and Buckley, C. (1997). Botulism. *Vet. Clin. N. Amer. Eq. Proc*. **13,** 107–128.

Wilkening, D.A. (2000). BCW attack scenarios. In: *The New Terror: Facing the Threat of Biological and Chemical Weapons* (eds. S.D. Drell, A.D. Sofaer and G.D. Wilson), pp. 76–114. Hoover Institution Press, Stanford University, Stanford, CA.

Williams, P. and Wallace, D. (1989). *Unit 731: Japan's Secret Biological Warfare in World War II*. The Free Press, New York, N.Y.

Wilson, R., Morris, J.G., Jr., Snyder, J.D. and Feldman, R.A. (1982). Clinical characteristics of infant botulism in the United States: a study of the non-California cases. *Pediatr. Infect. Dis*. **1,** 148–50.

Wright, S. (1990). Evolution of biological warfare policy, 1945–1990. In: *Preventing a Biological Arms Race* (ed. S. Wright), Chapter 2, pp. 26–68. The MIT Press, Cambridge, MA.

Zapor, M. and Fishbain, J.T. (2004). Aerosolized biologic toxins as agents of warfare and terrorism. *Respir. Care Clin*. **10,** 111–122.

Zilinskas, R.A. (1997) Iraq's biological weapons: the past and future? *JAMA* **278,** 418–424.

# Index

**Note:** Page numbers followed by *f* or *ff* indicate a figure or multiple figures will be found on that page, respectively